



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/86, 5/10, A61K 38/17 // C07K 14/705		A1	(11) International Publication Number: WO 96/26286 (43) International Publication Date: 29 August 1996 (29.08.96)
(21) International Application Number: PCT/US96/03041 (22) International Filing Date: 23 February 1996 (23.02.96)		(74) Agents: BAK, Mary, E. et al.; Howson and Howson, Spring House Corporate Center, P.O. Box 457, Spring House, PA 19477 (US).	
(30) Priority Data: 08/393,734 24 February 1995 (24.02.95) US		(81) Designated States: AL, AM, AU, BB, BG, BR, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KP, KR, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(60) Parent Application or Grant (63) Related by Continuation US Filed on 08/393,734 (CIP) 24 February 1995 (24.02.95)		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
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(54) Title: METHODS AND COMPOSITIONS FOR GENE THERAPY FOR THE TREATMENT OF DEFECTS IN LIPOPROTEIN METABOLISM			
(57) Abstract			
<p>The invention provides a recombinant viral vector comprising the DNA of, or corresponding to, at least a portion of the genome of an adenovirus, which portion is capable of infecting a hepatic cell; and a human <i>VLDL</i> receptor gene operatively linked to regulatory sequences directing its expression. The vector is capable of expressing the normal <i>VLDL</i> receptor gene product in hepatic cells <i>in vivo</i> or <i>in vitro</i>. This viral vector is useful in the treatment of metabolic disorders caused by the accumulation of <i>LDL</i> in plasma, such as familial hypercholesterolemia or familial combined hyperlipidemia.</p>			

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METHODS AND COMPOSITIONS FOR GENE THERAPY FOR THE
TREATMENT OF DEFECTS IN LIPOPROTEIN METABOLISM

This invention was supported by the National Institute of Health Grant Nos. DK 42193-05 and HD 29946.

5 The United States government has rights in this invention.

Field of the Invention

The present invention relates to the field of somatic gene therapy and the treatment of genetic 10 disorders related to lipoprotein metabolism.

Background of the Invention

The metabolism of lipids, particularly cholesterol, involves the interaction of a number of lipoproteins and apolipoproteins. Very low density 15 lipoprotein (VLDL) and apolipoprotein E (apoE) are key precursor molecules in the production of low density lipoprotein (LDL) and in the overall metabolism of lipids, including cholesterol. LDL is the major cholesterol-transport lipoprotein in human plasma.

20 The VLDL/apoE receptors are expressed in heart, skeletal muscle, and adipose tissue [F. M. Wittmaack et al, *Endocrinol.*, 136(1):340-348 (1995)] with lower levels of expression in the kidney, placenta, pancreas, and brain. This receptor has been suggested to play a role 25 in the uptake of triglyceride-rich lipoprotein particles by specific organs. The cDNA encoding the putative human VLDL receptor was recently cloned [M. E. Gafvels et al, *Som. Cell Mol. Genet.*, 19:557-569 (1993), incorporated by reference herein]. The receptor for LDL is located in 30 coated pits on the surfaces of cells in the liver and other organs.

As depicted in Fig. 1A, in a normal healthy human, the molecules apolipoprotein B48 (Apo-B48), apolipoprotein C-II (Apo-C-II) and Apo E form a

chylomicron particle in plasma passing through the intestines, which interacts with a chylomicron remnant receptor in the liver. After metabolism of the chylomicrons taken up by the remnant receptor, the liver produces the primary lipoprotein, VLDL, which contains Apo-E, Apo-C-II and apolipoprotein B100 (Apo B100). VLDL is metabolized into LDL, which binds to the LDL receptor in the liver via Apo B100. The LDL receptor in the liver facilitates the uptake of LDL by receptor-mediated endocytosis. LDL is degraded in lysosomes, and its cholesterol is released for metabolic use.

Defects in the metabolism of such lipoproteins and/or receptors result in several serious metabolic disorders. The human disease familial hypercholesterolemia (FH) is caused primarily by one or more mutations in the gene encoding the LDL receptor. FH is characterized clinically by (1) an elevated concentration of LDL; (2) deposition of LDL-derived cholesterol in tendons and skin (xanthomas) and in arteries (atheromas); and (3) inheritance as an autosomal dominant trait with a gene dosage effect. Individuals with FH develop premature coronary heart disease, usually in childhood. Heterozygotes number about 1 in 500 persons, placing FH among the most common inborn errors of metabolism.

Heterozygotes have twofold elevations in plasma cholesterol (350 to 550 mg/dl) from birth and tend to develop tendon xanthomas and coronary atherosclerosis after age 20. Homozygotes number 1 in 1 million persons and are characterized by severe hypercholesterolemia (650 to 1000 mg/dl), cutaneous xanthomas which appear within the first 4 years of life, and coronary heart disease which begins in childhood and frequently causes death before age 20. [J. Goldstein et al, "Familial Hypercholesterolemia", Chapter 48, in The Metabolic Basis of Inherited Disease, 6th ed., C. R. Scriver et al

(eds), McGraw-Hill Information Services Co., NY, NY, (1989) pp. 1215-1250].

Another metabolic disorder is familial combined hyperlipidemia (FCH) which was first associated with 5 hyperlipidemia in survivors of myocardial infarction and their relatives. FCH patients generally have one of three phenotypes: (1) elevated levels of *VLDL*, (2) elevated levels of *LDL*, or (3) increases in the levels of both lipoproteins in plasma. Unlike FH, FCH appears in 10 only 10 to 20 percent of patients in childhood, usually in the form of hypertriglyceridemia. Homozygosity for the trait may result in severe hypertriglyceridemia. [J. Goldstein et al, "Disorders of the Biogenesis and Secretion of Lipoproteins", Chapter 44B in The Metabolic 15 Basis of Inherited Disease, 6th ed., C. R. Scriver et al (eds), McGraw-Hill Information Services Co., NY, NY, (1989) pp. 1155-1156]. This disorder is also associated with the appearance of glucose intolerance and obesity in a number of individuals.

20 The most striking abnormality of FCH is marked elevation of *VLDL* content of plasma. Increased production of *VLDL* leads to an expanded plasma pool of *VLDL* in some individuals, but in others with more efficient lipolysis, it results in increased levels of 25 *LDL*. FCH is characterized by an excess production of *LDL*, rather than a genetic defect in the *LDL* receptor. The *LDL* receptors of cultured fibroblasts appear to be normal in FCH patients.

Clinical experience suggests that FCH is at 30 least five times as prevalent as FH, occurring in about 1 percent of the North American population. The predilection toward coronary artery disease among patients with this disorder makes it the most prominent known metabolic cause of premature atherosclerosis [J. 35 Goldstein et al, cited above].

When LDL receptors are deficient as in FH (see Fig. 1B), or excess LDL is produced due to excess VLDL as in FCH, the efficient removal of LDL from plasma by the liver declines, and the level of LDL rises in inverse proportion to the receptor number. The excess plasma LDL is deposited in connective tissues and in scavenger cells, resulting in the symptoms of either disorder.

Presently, treatment for FH and FCH is directed at lowering the plasma level of LDL by the administration of drugs, i.e., combined administration of a bile acid-binding resin and an inhibitor of 3-hydroxy-3-methylglutaryl CoA reductase for treatment of FH and niacin for treatment of FCH. However, FH homozygotes with two nonfunctional genes are resistant to drugs that work by stimulating LDL receptors. Similarly, such drugs are not particularly effective in FCH. In FH homozygotes, plasma LDL levels can be lowered only by physical or surgical means.

Administration of normal LDL receptor genes by gene therapy using an adenovirus vector has been contemplated for the treatment of FH. Adenovirus vectors are capable of providing extremely high levels of transgene delivery to virtually all cell types, regardless of the mitotic state. The efficacy of this system in delivering a therapeutic transgene *in vivo* that complements a genetic imbalance has been demonstrated in animal models of various disorders [K. F. Kozarsky et al, Somatic Cell Mol. Genet., 19:449-458 (1993) ("Kozarsky I"); K. F. Kozarsky et al, J. Biol. Chem., 269:13695-13702 (1994) ("Kozarsky II"); Y. Watanabe, Atherosclerosis, 36:261-268 (1986); K. Tanzawa et al, FEBS Letters, 118(1):81-84 (1980); J.L. Golasten et al, New Engl. J. Med., 309:288-296 (1983); S. Ishibashi et al, J. Clin. Invest., 92:883-893 (1993); and S. Ishibashi et al, J. Clin. Invest., 93:1885-1893 (1994)]. The use

of adenovirus vectors in the transduction of genes into hepatocytes *in vivo* has previously been demonstrated in rodents and rabbits [see, e.g., Kozarsky II, cited above, and S. Ishibashi et al, J. Clin. Invest., 92:883-893
5 (1993)].

Recent research has shown that introduction of a recombinant adenovirus encoding the human *LDL* receptor ("LDLR") cDNA into the livers of *LDL* receptor-deficient Watanabe heritable hyperlipidemic (WHHL) rabbits, which 10 mimic the condition of FH, resulted in large, transient reductions in plasma cholesterol. The transient nature of the effect of recombinant adenoviruses in most situations is attributed to the development of cellular immune responses to the virus-infected cells and their 15 subsequent elimination. Antigenic targets for immune mediated clearance are viral proteins expressed from the recombinant viral genome and/or the product of the transgene, which in this case, is the *LDL* receptor protein [Y. Yang et al, Proc. Natl. Acad. Sci., USA,
20 91:4407-4411 (May 1994); Y. Yang et al, Immun., 1:433-442 (August 1994)].

Additionally, repeated reinfusions of the *LDLR* gene-containing adenovirus did not produce similar, subsequent cholesterol reductions due to the development 25 of neutralizing anti-adenovirus antibodies [Kozarsky I and Kozarsky II, cited above; see also Y. Yang et al, Immun., 1:433-442 (August 1994), all incorporated by reference herein].

There remains a need in the art for therapeutic 30 compositions and gene therapy strategies which enable effective treatment and/or prevention of FH and FCH, as well as other defects in lipoprotein metabolism.

Summary of the Invention

In one aspect, the invention provides a recombinant viral vector comprising the DNA of, or corresponding to, at least a portion of the genome of an adenovirus, which portion is capable of infecting a hepatic cell; and a human VLDL receptor ("VLDLR") gene operatively linked to regulatory sequences directing its expression, the vector capable of expressing the VLDLR gene product in the hepatic cell *in vivo* or *in vitro*.

In another aspect, the invention provides a mammalian cell infected with the viral vector described above.

In still a further aspect, the invention provides a method for delivering and stably integrating a VLDLR gene into the chromosome of a mammalian hepatocyte cell comprising introducing into said cell an effective amount of a recombinant viral vector described above.

Another aspect of this invention is a method for treating a patient having a metabolic disorder comprising administering to the patient by an appropriate route an effective amount of an above described vector containing a normal VLDLR gene, wherein said VLDLR gene is integrated into the chromosome of said patient's hepatocytes and said receptor is expressed stably *in vivo* at a location in the body where it is not normally expressed.

Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

30 Brief Description of the Drawings

Fig. 1A is a schematic drawing of normal human and rabbit lipoprotein metabolism. The apolipoproteins are referred to as B48, B100, C-II, and E. LDL and VLDL are identified.

Fig. 1B is a schematic drawing of lipoprotein metabolism in FH patients and WHHL rabbits. The abbreviations are as described in Fig. 1A.

5 Fig. 1C is a schematic drawing of lipoprotein metabolism in rabbits infused with the recombinant VLDLR gene according to the invention.

10 Fig. 2 is a schematic drawing of plasmid pAd.CMVVLDLR, which contains adenovirus map units 0-1 (Ad 0-1), followed by a cytomegalovirus enhancer/promoter (CMV enh/prom), a human VLDLR gene, a polyadenylation signal (pA), adenovirus map units 9-16 (Ad 9-16) and plasmid sequences from plasmid pAT153 including an origin of replication and ampicillin resistance gene.
15 Restriction endonuclease enzymes are represented by conventional designations in the plasmid construct.

20 Fig. 3 is a schematic map of recombinant adenovirus H5.010CMVVLDLR, in which 0 to 100 represent the map units of an adenovirus type 5 (Genbank Accession No. M73260), and the CMV/VLDLR/pA minicassette of pAd.CMVVLDLR is inserted between adenovirus map units 1 and 9, with the remaining Ad5 map units 9-100 having a partial E3 gene deletion between about map unit 78.5 and about 84.3.

25 Fig. 4A is a graph plotting changes in plasma cholesterol levels in mg/dl for WHHL rabbits as a function of days before and after receiving recombinant adenovirus H5.010CMVlacZ. The symbols represent individual animals. See Example 3.

30 Fig. 4B is a graph plotting changes in plasma cholesterol levels in mg/dl for WHHL rabbits as a function of days before and after receiving recombinant adenovirus H5.010CMVVLDLR. The symbols represent the response of four individual animals. See Example 3.

Fig. 5 is a bar graph representing cholesterol levels (measured as % pre-infusion) in mice infused with recombinant adenovirus H5.010CMVlacZ (lacZ), recombinant adenovirus H5.010CMVLDLR and recombinant adenovirus H5.010CBhLDLR. The dotted bars represent pre-infusion levels and the solid bars represent post-infusion levels. See Example 4.

Fig. 6 is a bar graph representing cholesterol levels, specifically the levels of the fractions of plasma lipoproteins (measured as mg/fraction) in mice infused with recombinant adenovirus H5.010CMVlacZ (lacZ), recombinant adenovirus H5.010CMVLDLR and recombinant adenovirus H5.010CBhLDLR. The solid bars represent proteins or fragments falling within a density (d) $>$ 1.21; the thickly cross-hatched bars represent HDL; the closely cross-hatched bars represent LDL, the spaced apart slanted hatched bars represent intermediate density lipoprotein (IDL), and the clear bars represent VLDL levels. See Example 4.

Fig. 7A is a graph plotting changes in cholesterol levels (measured in mg/dl) as a function of days pre- and post-infusion for mice infused with H5.010CMVlacZ. The symbols represent the responses of individual animals. See Example 5.

Fig. 7B is a graph plotting changes in cholesterol levels (measured in mg/dl) as a function of days pre- and post-infusion for mice infused with H5.010CBhLDLR. The symbols are the same as for Fig. 7A. See Example 5.

Fig. 7C is a graph plotting changes in cholesterol levels (measured in mg/dl) vs. days pre and post-infusion for mice infused with H5.010CMVLDLR. The symbols are the same as for Fig. 7A. See Example 5.

Fig. 7D is a graph providing the average results \pm standard deviation from two experiments for mice infused with H5.010CMVLacZ (n=9) or with H5.010CMVVLDR (n=10). Average pre-infusion cholesterol levels were 870 mg/dl and 946 mg/dl, respectively. Asterisks indicate $p < 0.05$.

Figs. 8A-8F are the DNA sequence [SEQ ID NO: 1] with encoded amino acid sequence [SEQ ID NO: 2] of the human VLDL receptor gene, as reported by Gafvels et al, cited above.

Figs. 9A-9I are the DNA sequence of pAd.CMVVLDR [SEQ ID NO: 3], in which Ad 0-1 spans nucleotides 12-364, CMV ehn/prom spans nucleotides 381-862; nucleotides 966-4107 encode VLDR, pA spans nucleotides 4192-4390; Ad 9.2-16.1 span nucleotides 4417-6880 and nucleotides 6881-9592 are pAT153 sequences.

Fig. 10A is a bar chart illustrating the CTL activity (average \pm standard deviation) measured at an effector:target cell ratio of 25:1. ** = $p < 0.005$; * = $p < 0.05$.

Fig. 10B is a line graph illustrating the CTL activity measured against varying effector:target ratios.

Fig. 11A is a graph summarizing neutralizing antibody titer present in BAL samples of C57BL/6 mice adenovirus-infected on day 0 and necrotized on day 28 as described in Example 9. Control represents normal mice ("control"); CD4 mAB represents CD4 $^{+}$ cell depleted mice; IL-12 represents IL-12 treated mice and IFN- γ represent IFN- γ treated mice.

Fig. 11B is a graph summarizing the relative amounts (OD_{405}) of IgG present in BAL samples. The symbols are as described in Fig. 11A.

Fig. 11C is a graph summarizing the relative amounts (OD_{405}) of IgA present in BAL samples. The symbols are as described in Fig. 11A.

Detailed Description of the Invention

The present invention provides novel compositions and methods which enable the therapeutic treatment of metabolic disorders, such as FH and FCH, characterized by the accumulation of *LDL* in human plasma. This invention provides for the use of a viral vector to introduce and stably express a gene normally expressed in mammals, i.e., the gene encoding a normal receptor for very low density lipoprotein (*VLDLR*), in a location in the body where that gene is not naturally present, i.e., in the liver.

The methods and compositions of the present invention overcome the problems previously identified in the gene therapy treatment of *LDL* receptor-deficient individuals. As described in detail below, by use of a viral vector capable of targeting cells of the liver, the *VLDL* receptor gene is introduced into and stably expressed in liver cells. The present invention differs from direct gene replacement in that the *VLDL* receptor protein is expressed normally in *LDL* receptor deficient individuals, e.g., the macrophages. Thus, gene therapy using a liver-directed viral vector carrying a *VLDLR* gene would result not in expression of a new gene product, but rather, in *de novo* expression in an organ which otherwise does not express the gene product. Importantly, the patient does not mount an immune response against the *VLDLR* gene product expressed in the liver because the vector-delivered *VLDLR* gene is not recognized as a foreign antigen, and there is no induction of CTL-mediated elimination of the transfected cell. In contrast, CTL-mediated elimination of viral vectors is a problem when an *LDLR* gene is administered to an *LDLR*-deficient individual with FH [see, e.g., Kozarsky I and II, cited above].

Due to this recognition of the *VLDLR* gene by the patient's immune system as a known gene, and to the tendency of hepatocytes to have a long life in circulation, the hepatocytes transfected with the vector of this invention, which express the *VLDLR* gene, tend to be stable and *VLDLR* expression is not transient. *VLDLR* gene expression in transfected hepatocytes occurs for the duration of the hepatocyte's life. The lipoprotein metabolic disorder may be treated for longer times without the need for reinfusing the viral vector, thus limiting the number of viral exposures and potential immune reactions to vector-encoded viral proteins.

The vectors and methods of this invention can provide gene therapy useful to treat and/or supplement current treatments for lipoprotein metabolic disorders. The presence of the *VLDL* receptor gene in the transfected hepatocytes according to this invention permits the binding of *VLDL*, a precursor of *LDL*, from the plasma at the site of the liver, thereby decreasing the amount of *VLDL* in plasma. The decrease in *VLDL* in the plasma consequently decreases the production of plasma *LDL*.

For example, in FH, this reduction in plasma *LDL* can compensate for the defective *LDL* receptors in the liver. In FCH, this reduced production of plasma *LDL* from *VLDL* prevents the normal *LDL* receptors in the liver from becoming overloaded by excess *LDL*, and reduces the excess *VLDL* which contributes to the disorder. Compare, for example, the schematic representations of the normal operation of lipid metabolism (Fig. 1A) to the abnormal metabolism caused by FH (Fig. 1B) and then to the method of this invention (Fig. 1C).

I. **Recombinant Viral Particles as Gene Therapy Vectors**

The compositions of this invention involve the construction of desirable gene therapy vectors, which are capable of delivering and stably integrating a functional, normal *VLDL* receptor gene to hepatocytes. Such gene therapy vectors include a selected virus vector, desirably deleted in one or more viral genes, a minigene containing the *VLDLR* gene under the control of regulatory sequences, and optional helper viruses and/or packaging cell lines which supply to the viral vectors any necessary products of deleted viral genes.

The viral sequences used in the vectors, helper viruses, if needed, and recombinant viral particles, and other vector components and sequences employed in the construction of the vectors described herein are obtained from commercial or academic sources based on previously published and described sequences. These viral materials may also be obtained from an individual patient. The viral sequences and vector components may be generated by resort to the teachings and references contained herein, coupled with standard recombinant molecular cloning techniques known and practiced by those skilled in the art. Modifications of existing nucleic acid sequences forming the vectors, including sequence deletions, insertions, and other mutations taught by this specification may be generated using standard techniques.

The methods employed for the selection of viral sequences useful in a vector, the cloning and construction of *VLDLR* "minigene" and its insertion into a desired viral vector and the production of a recombinant infectious viral particle by use of helper viruses and the like are within the skill in the art given the teachings provided herein.

A. Construction of the "Minigene"

By "minigene" is meant the combination of the *VLDLR* gene and the other regulatory elements necessary to transcribe the gene and express the gene product *in vivo*. The human *VLDL* receptor sequence has been provided [see, Gafvels et al, cited above; SEQ ID NOS: 1 and 2]. Generally, the entire coding region of this receptor sequence is used in the minigene; the 5' and 3' untranslated sequences of SEQ ID NO: 1 are not essential to the minigene. *VLDL* receptor genes of other mammalian origins, e.g., rabbit, monkey, etc., may also be useful in this invention.

The *VLDL* receptor gene (*VLDLR*) is operatively linked to regulatory components in a manner which permits its transcription. Such components include conventional regulatory elements necessary to drive expression of the *VLDLR* transgene in a cell transfected with the viral vector. Thus the minigene also contains a selected promoter which is linked to the transgene and located, with other regulatory elements, within the selected viral sequences of the recombinant vector.

Selection of the promoter is a routine matter and is not a limitation of this invention. Useful promoters may be constitutive promoters or regulated (inducible) promoters, which will enable control of the amount of the transgene to be expressed. For example, a desirable promoter is that of the cytomegalovirus immediate early promoter/enhancer [see, e.g., Boshart et al, Cell, 41:521-530 (1985)]. Another desirable promoter includes the Rous sarcoma virus LTR promoter/enhancer. Still another promoter/enhancer sequence is the chicken cytoplasmic β -actin promoter [T. A. Kost et al, Nucl. Acids Res., 11(23):8287 (1983)]. Other suitable promoters may be selected by one of skill in the art.

The minigene may also desirably contain nucleic acid sequences heterologous to the viral vector sequences including sequences providing signals required for efficient polyadenylation of the transcript (poly-A or pA) and introns with functional splice donor and acceptor sites. A common poly-A sequence which is employed in the exemplary vectors of this invention is that derived from the papovavirus SV-40. The poly-A sequence generally is inserted in the minigene following the transgene sequences and before the viral vector sequences. A common intron sequence is also derived from SV-40, and is referred to as the SV-40 T intron sequence. A minigene of the present invention may also contain such an intron, desirably located between the 5 promoter/enhancer sequence and the transgene. Selection 10 of these and other common vector elements are conventional [see, e.g., Sambrook et al, "Molecular Cloning. A Laboratory Manual.", 2d ed., Cold Spring Harbor Laboratory, New York (1989) and references cited 15 therein] and many such sequences are available from 20 commercial and industrial sources as well as from Genbank.

As stated above, the minigene is located in the site of any selected deletion in the viral vector. 25 See Example 1 below.

B. Construction of The Viral Plasmid Vector

Although a number of viral vectors have been suggested for gene therapy, the most desirable vector for this purpose is a recombinant adenoviral 30 vector or adeno-associated vector. Adenovirus vectors as described below are preferred because they can be purified in large quantities and highly concentrated, and the virus can transduce genes into non-dividing cells.

However, it is within the skill of the art for other adenovirus, or even retrovirus, vaccinia or other virus vectors to be similarly constructed.

Adenoviruses are eukaryotic DNA viruses that can be modified to efficiently deliver a therapeutic or reporter transgene to a variety of cell types. Human adenoviruses comprise a linear, approximately 36 kb double-stranded DNA genome, which is divided into 100 map units (m.u.), each of which is 360 bp in length. The DNA contains short inverted terminal repeats (ITR) at each end of the genome that are required for viral DNA replication. The gene products are organized into early (E1 through E4) and late (L1 through L5) regions, based on expression before or after the initiation of viral DNA synthesis [see, e.g., Horwitz, Virology, 2d edit., ed. B. N. Fields, Raven Press, Ltd. , New York (1990)]. The general adenoviruses types 2 and 5 (Ad2 and Ad5, respectively), are not associated with human malignancies.

Suitable adenovirus vectors useful in gene therapy are well known [see, e.g., M. S. Horwitz et al, "Adenoviridae and Their Replication", Virology, second edition, pp. 1712, ed. B. N. Fields et al, Raven Press Ltd., New York (1990); M. Rosenfeld et al, Cell, 68:143-155 (1992); J. F. Engelhardt et al, Human Genet. Ther., 4:759-769 (1993); Y. Yang et al, Nature Genet., 7:362-269 (1994); J. Wilson, Nature, 365:691-692 (Oct. 1993); B. J. Carter, in "Handbook of Parvoviruses", ed. P. Tijssser, CRC Press, pp. 155-168 (1990). The selection of the adenovirus type is not anticipated to limit the following invention.

Adenovirus vectors useful in this invention may include the DNA sequences of a number of adenovirus types. The adenovirus sequences useful in the vectors described herein may be obtained from any known

adenovirus type, including the presently identified 41
human types [see, e.g., Horwitz, cited above]. The
sequence of a strain of adenovirus type 5 may be readily
obtained from Genbank Accession No. M73260. Similarly,
5 adenoviruses known to infect other animals may also be
employed in the vector constructs of this invention. A
variety of adenovirus strains are available from the
American Type Culture Collection, Rockville, Maryland, or
available by request from a variety of commercial and
10 institutional sources.

Adenovirus vectors useful in this
invention include recombinant, defective adenoviruses,
optionally bearing other mutations, e.g., temperature-
sensitive mutations, deletions and hybrid vectors formed
15 with adenovirus/adeno-associated virus sequences.
Suitable vectors are described in the published
literature [see, for example, Kozarsky I and II, cited
above, and references cited therein, U. S. Patent No.
5,240,846 and the co-pending applications incorporated
20 herein by reference below.

Useful adenovirus vectors for delivery of
the VLDLR gene to the liver, minimal adenovirus nucleic
acid sequences may be used to make a vector, in which
case the use of a helper virus to produce a hybrid virus
25 particle is required. Alternatively, only selected
deletions of one or more adenovirus genes may be employed
to construct a viral vector. Deleted gene products can
be supplied by using a selected packaging cell line which
supplies the missing gene product.

30 1. Recombinant Minimal Adenovirus
Desirable adenovirus (Ad) vectors
useful in the present invention are described in detail
in co-pending, co-owned U.S. Patent Application Serial
No. 08/331,381, which is incorporated by reference herein
35 for the purpose of describing these vectors.

Briefly summarized, the minimal Ad virus is a viral particle containing only the adenovirus cis-elements necessary for replication and virion encapsidation, but otherwise deleted of all adenovirus genes. That is, the vector contains only the cis-acting 5' and 3' inverted terminal repeat (ITR) sequences of an adenovirus (which function as origins of replication) and the native 5' packaging/enhancer domain, that contains sequences necessary for packaging linear Ad genomes and enhancer elements for the E1 promoter. This left terminal (5') sequence of the Ad5 genome spans bp 1 to about 360 of the conventional published Ad5 adenovirus genome, also referred to as map units 0-1 of the viral genome, and generally is from about 353 to about 360 nucleotides in length. This sequence includes the 5'ITR (bp 1 to about 103 of the adenovirus genome); and the packaging/enhancer domain (bp about 194 to about 358 of the adenovirus genome). The minimal 3' adenovirus sequences of the adenovirus vector may include the right terminal (3') ITR sequence of the adenoviral genome spanning about bp 35,353 to the end of the adenovirus genome, or map units -98.4-100. This sequence is generally about 580 nucleotide in length. Between such sequences, a VLDLR minigene, as described above, is inserted.

Production of an infectious particle from this minimal Ad viral vector involves the assistance of a helper virus, as discussed below. A second type of minimal vector also disclosed in the above-incorporated reference places the 5' Ad terminal sequence in a head-to-tail arrangement relative to the 3' terminal sequence. The minimal Ad vector co-infected with a helper virus and/or a packaging cell line provides all of the viral gene products necessary to produce an infective recombinant viral particle containing the VLDLR minigene.

Alternatively, this vector can contain additional adenovirus gene sequences, which then are not required to be supplied by a helper virus.

2. Other Defective Adenoviruses

5 Recombinant, replication-deficient adenoviruses useful for gene therapy of this invention may be characterized by containing more than the minimal adenovirus sequences defined above. These other Ad vectors can be characterized by deletions of various 10 portions of gene regions of the virus, and infectious virus particles formed by the optional use of helper viruses and/or packaging cell lines. Suitable defective adenoviruses are described in more detail in Kozarsky and Wilson, Curr. Opin. Genet. Devel., 3:499-503 (1993); 15 Kozarsky I and II, cited above, and references cited therein, all incorporated herein by reference.

As one example, suitable vectors may be formed by deleting all or a sufficient portion of the adenoviral early immediate early gene E1a (which spans mu 20 1.3 to 4.5) and delayed early gene E1b (which spans mu 4.6 to 11.2) so as to eliminate their normal biological functions. These replication-defective E1-deleted viruses are capable of replicating and producing 25 infectious virus when grown on an adenovirus-transformed, complementation human embryonic kidney cell line, the 293 cell [ATCC CRL1573], containing functional adenovirus E1a and E1b genes which provide the corresponding gene products in trans. The resulting virus is capable of infecting many cell types and can express a transgene 30 (i.e., VLDLR gene), but cannot replicate in most cells that do not carry the E1 region DNA unless the cell is infected at a very high multiplicity of infection. Extensive experience in animals indicates that E1-deleted vectors are not particularly desirable for gene therapy

because low levels of viral proteins are expressed which elicit destructive cellular immune responses.

As a preferred example, all or a portion of the adenovirus delayed early gene E3 (which spans mu 76.6 to 86.2) may be eliminated from the adenovirus sequence which forms a part of the hybrid construct. The function of E3 is irrelevant to the function and production of the recombinant virus particle. For example, Ad vectors may be constructed with a therapeutic minigene inserted into the E1-deleted region of the known mutant Ad5 sub360 backbone [J. Logan et al, Proc. Natl. Acad. Sci. USA, 81:3655-3659 (1984)]; or the Ad5 mutant dl7001 backbone [Dr. William Wold, Washington University, St. Louis]. Both mutant viruses also contain a deletion in the E3 region of the adenoviral genome; in sub360, at 78.5 to 84.3 mu, and in dl7001, at 78.4 to 86 mu. The life cycle of both sub360 and dl7001 display wild type characteristics.

More preferred adenovirus vectors may be constructed having a deletion of the E1 gene, at least a portion of the E3 region, and an additional deletion within adenovirus genes other than E1 and E3 to accommodate the VLDLR minigene and/or other mutations which result in reduced expression of adenoviral protein and/or reduced viral replication. For example, all or a portion of the adenovirus delayed early gene E2a (which spans mu 67.9 to 61.5) may be eliminated from the adenovirus vector. It is also anticipated that portions of the other delayed early genes E2b (which spans mu 29 to 14.2) and E4 (which spans mu 96.8 to 91.3) may also be eliminated from the adenovirus vector.

Deletions may also be made in any of the late genes L1 through L5, which span mu 16.45 to 99 of the adenovirus genome. Similarly, deletions may be useful in the intermediate genes IX (which maps between

mu 9.8 and 11.2) and IVa, (which maps betw en 16.1 to 11.1). Other useful deletions may also be made in the other structural or non-structural adenovirus genes.

An adenovirus sequence for use in the present invention may contain deletions of E1 only. Alternatively, deletions of entire genes or portions effective to destroy their biological activity may be used in any combination. For example, in one exemplary vector, the adenovirus sequence may contain deletions of the E1 genes and the E3 gene, or of the E1, E2a and E3 genes, or of the E1 and E4 genes, or of E1, E2a and E4 genes, with or without deletion of E3, and so on.

Vectors may also contain additional mutations in genes necessary for viral replication.

Adenovirus vectors may contain a mutation which produces temperature-sensitive (ts) viruses. Among such mutations include the incorporation of the missense temperature-sensitive mutation in the E2a region found in the Ad5 H5ts125 strain [P. Vander Vliet et al, J. Virol., 15:348-354 (1975)] at 62.5 mu. A single amino acid substitution (62.5 mu) at the carboxy end of the 72 kd protein (DBP) produced from the E2a gene in this strain produces a protein product which is a single-stranded DNA binding protein and is involved in the replication of adenoviral genomic DNA. At permissive temperatures (approximately 32°C) the ts strain is capable of full life cycle growth on HeLa cells, while at non-permissive temperatures (approximately 38°C), no replication of adenoviral DNA is seen. In addition, at non-permissive temperatures, decreased immunoreactive 72 kd protein is seen in HeLa cells.

Exemplary vectors for use in this invention, for example, may be obtained by combining

fragments from three independent DNA constructs, including sub360 or dl7001, H5ts125, and a cDNA plasmid with E1a sequences placed 5' to a therapeutic minigene. This type of vector is described, for example, by J. F. Engelhardt et al, Proc. Natl. Acad. Sci. USA, 91:6196-6200 (June 1994); Y. Yang et al, Nature Genet., 7: 362-369 (July, 1994) and references cited therein, all references incorporated herein by reference. Due to the mutations in the vector, there is reduced viral replication, reduction in expressed protein and an increase in the persistence of transgene expression. Other preferred adenovirus vectors contain the H5ts125 mutation in addition to E3 deletions of sub360 and dl7001. The minigene containing VLDLR as the transgene may be inserted into any deleted region of the selected Ad virus.

An exemplary Ad virus vector used to demonstrate this invention is the defective adenovirus vector H5.010CMVVLDLR, which contains adenovirus sequences Ad m.u. 0-1, followed by a VLDLR minigene, and the sequence Ad m.u.9 to 100 with small deletions in E3. See Fig. 3, described above. The recombinant adenovirus was fully deleted of E1a, E1b and partially deleted of E3. This recombinant virus vector is described in detail in Example 1.

3. Ad/AAV Hybrid Vectors

Another preferred vector is a hybrid Ad/AAV vector, which is the subject of co-owned, co-pending U.S. Patent Application Ser. No. 08/331,384, which is incorporated by reference herein.

At a minimum, the adenovirus nucleic acid sequences employed in the hybrid vector of this invention are the minimal adenovirus genomic sequences required for packaging adenoviral genomic DNA into a preformed capsid head, as described above. The entire

adenovirus 5' sequence containing the 5' ITR and packaging/enhancer region can be employed as the 5' adenovirus sequence in the hybrid vector. The 3' adenovirus sequences of the vector include the right 5 terminal (3') ITR sequence of the adenoviral genome discussed above. Some modifications to these sequences which do not adversely affect their biological function may be acceptable.

Also part of the hybrid vectors of this invention are sequences of an adeno-associated virus. The AAV sequences useful in the hybrid vector are the viral sequences from which the rep and cap polypeptide encoding sequences are deleted. More specifically, the AAV sequences employed are the cis-acting 5' and 3' inverted terminal repeat (ITR) sequences [See, e.g., B. J. Carter, cited above]. The AAV ITR sequences are about 143 bp in length. Substantially the entire sequences encoding the ITRs are used in the vectors, although some degree of minor modification of these sequences is expected to be permissible for this use. The ability to modify these ITR sequences is within the skill of the art. See, e.g., Sambrook et al, cited above.

In the Ad/AAV hybrid vector construct, the AAV sequences are flanked by the adenovirus sequences discussed above. The 5' and 3' AAV ITR sequences themselves flank a VLDLR minigene sequence as described above. Thus, the sequence formed by the VLDLR minigene and flanking 5' and 3' AAV sequences may be inserted at any deletion site in the adenovirus sequences of the vector. For example, the AAV sequences are desirably inserted at the site of deleted E1a/E1b genes of the adenovirus, i.e., after map unit 1. Alternatively, the AAV sequences may be inserted at an E3 deletion, E2a deletion, and so on. If only the

adenovirus 5' ITR/packaging sequences and 3' ITR sequences are used in the vector, the AAV sequences are inserted between them.

As described above for the minimum adenovirus sequences, those gene sequences not present in the adenovirus portion of the hybrid vector must be supplied by either a packaging cell line and/or a helper adenovirus to generate the recombinant hybrid viral particle. Uptake of this hybrid virus by the cell is caused by the infective ability contributed to the vector by the adenovirus and AAV sequences. Once the virus or virus conjugate is taken up by a cell, the AAV ITR flanked transgene must be rescued from the parental adenovirus backbone. Rescue of the transgene is dependent upon supplying the infected cell with an AAV rep gene.

The AAV rep gene can be supplied to the hybrid virus by several methods described in the above-incorporated application. One embodiment for providing rep proteins in trans is by transfecting into the target monolayer of cells previously infected with the hybrid vector, a liposome enveloped plasmid containing the genes encoding the AAV rep 78 kDa and 52 kDa proteins under the control of the AAV P5 promoter. More preferably for in vivo use, the AAV rep gene may also be delivered as part of the hybrid virus. One embodiment of this single particle concept is supplied by a polycation conjugate of hybrid virus. Infection of this modified virus conjugate is accomplished in the same manner and with regard to the same target cells as identified above. However, the polylysine conjugate of the hybrid virus onto which was directly complexed a plasmid that encoded the rep 78 and 52 proteins, combines all of the functional components into a single particle structure. Thus, the hybrid virus conjugate permits

delivery of a single particle to the cell, which is considerably more desirable for therapeutic use. In another embodiment, the hybrid virus is modified by cloning the *rep* cDNA directly into the adenovirus genome 5 portion of the hybrid vector.

These and additional aspects of this hybrid vector are provided by the above-incorporated by reference application.

C. Production of the Recombinant Viral

10 Particle

1. Helper Viruses/Packaging Cell Lines

Depending upon the adenovirus gene content of the plasmid vectors employed to carry the VLDLR minigene, a packaging cell line or a helper 15 adenovirus or both may be necessary to provide sufficient adenovirus gene sequences necessary to produce an infective recombinant viral particle containing the VLDLR minigene.

Useful helper viruses contain 20 selected adenovirus gene sequences not present in the adenovirus vector construct or expressed by the cell line in which the vector is transfected. A preferred helper virus is desirably replication defective and contains a variety of adenovirus genes in addition to the modified 25 sequences described above. In this setting, the helper virus is desirably used in combination with a packaging cell line that stably expresses adenovirus genes. Helper viruses may also be formed into poly-cation conjugates as described in Wu et al, J. Biol. Chem., 30 264:16985-16987 (1989); K. J. Fisher and J. M. Wilson, Biochem. J., 299:49 (April 1, 1994), and in U. S. Patent Application Serial No. 08/331,381, incorporated by reference herein.

Helper virus may optionally contain a 35 second reporter minigene. A number of such reporter

genes are known to the art. The presence of a reporter gene on the helper virus which is different from the transgene on the adenovirus vector allows both the Ad vector and the helper virus to be independently monitored. This second reporter is used to enable separation between the resulting recombinant virus and the helper virus upon purification. The construction of desirable helper cells is within the skill of the art.

As one example, if the cell line employed to produce the viral vector is not a packaging cell line, and the vector contains only the minimum adenovirus sequences identified above, the helper virus may be a wild type Ad vector supplying the necessary adenovirus early genes E1, E2a, E4 and all remaining late, intermediate, structural and non-structural genes of the adenovirus genome. However, if, in this situation, the packaging cell line is 293, which supplies the E1 proteins, the helper cell line need not contain the E1 gene.

In another embodiment, if the adenovirus vector construct is replication defective (no E1 gene and optionally no E3 gene) and the 293 cell line is employed, no helper virus is necessary for production of the hybrid virus. E3 may be eliminated from the helper virus because this gene product is not necessary for the formation of a functioning virus particle.

Preferably, to facilitate purification and reduce contamination of the viral vector particle with the helper virus, it is useful to modify the helper virus' native adenoviral gene sequences which direct efficient packaging, so as to substantially disable or "cripple" the packaging function of the helper virus or its ability to replicate.

A desirable "crippled" adenovirus is modified in its 5' ITR packaging/enhancer domain, which

normally contains at least seven distinct yet functionally redundant sequences necessary for efficient packaging of replicated linear adenovirus genomes ("PAC" sequences). Within a stretch of nucleotide sequence from 5 bp 194-358 of the Ad5 genome, five of these PAC sequences are localized: PAC I or its complement at bp 241-248 [SEQ ID NO: 4], PAC II or its complement at bp 262-269 [SEQ ID NO: 5], PAC III or its complement at bp 304-311 [SEQ ID NO: 6], PAC IV or its complement at bp 314-321 [SEQ ID NO: 7], and PAC V or its complement at bp 339-346 [SEQ ID NO: 8].

Mutations or deletions may be made to one or more of these PAC sequences in an adenovirus helper virus to generate desirable crippled helper viruses. Modifications of this domain may include 5' adenovirus sequences which contain less than all five of the native adenovirus PAC sequences, including deletions of contiguous or non-contiguous PAC sequences. An alternative modification may be the replacement of one or 10 more of the native PAC sequences with one or more repeats of a consensus sequence containing the most frequently used nucleotides of the five native PAC sequences. Alternatively, this adenovirus region may be modified by 15 deliberately inserted mutations which disrupt one or more of the native PAC sequences. One of skill in the art may further manipulate the PAC sequences to similarly achieve 20 the effect of reducing the helper virus packaging efficiency to a desired level.

It should be noted that one of skill 25 in the art may design other helper viruses or develop other packaging cell lines to complement the adenovirus deletions in the vector construct and enable production of the recombinant virus particle, given this information. Therefore, the use or description of any

particular helper virus or packaging cell line is not limiting.

In the presence of other packaging cell lines which are capable of supplying adenoviral proteins in addition to the E1, the helper virus may accordingly be deleted of the genes encoding these adenoviral proteins. Such additionally deleted helper viruses also desirably contain crippling modifications as described above.

Poly-cation helper virus conjugates, which may be associated with a plasmid containing other adenoviral genes, which are not present in the helper virus may also be useful. The helper viruses described above may be further modified by resort to adenovirus-polylysine conjugate technology. See, e.g., Wu et al, cited above; and K. J. Fisher and J. M. Wilson, cited above.

Using this technology, a helper virus containing preferably the late adenoviral genes is modified by the addition of a poly-cation sequence distributed around the capsid of the helper virus. Preferably, the poly-cation is poly-lysine, which attaches around the negatively-charged vector to form an external positive charge. A plasmid is then designed to express those adenoviral genes not present in the helper virus, e.g., the E1, E2 and/or E4 genes. The plasmid associates to the helper virus-conjugate through the charges on the poly-lysine sequence. This conjugate permits additional adenovirus genes to be removed from the helper virus and be present on a plasmid which does not become incorporated into the virus during production of the recombinant viral vector. Thus, the impact of contamination is considerably lessened.

2. Assembly of Viral Particle and
Infection of a Cell Line

Assembly of the selected DNA

sequences of the adenovirus, the AAV and the reporter genes or therapeutic genes and other vector elements into the hybrid vector and the use of the hybrid vector to produce a hybrid viral particle utilize conventional techniques. Such techniques include conventional cloning techniques of cDNA such as those described in texts [Sambrook et al, cited above], use of overlapping oligonucleotide sequences of the adenovirus and AAV genomes, polymerase chain reaction, and any suitable method which provides the desired nucleotide sequence. Standard transfection and co-transfection techniques are employed, e.g., CaPO₄ transfection techniques using the complementation 293 cell line. Other conventional methods employed include homologous recombination of the viral genomes, plaquing of viruses in agar overlay, methods of measuring signal generation, and the like.

For example, following the construction and assembly of the desired minigene-containing plasmid vector, the vector is infected *in vitro* in the presence of an optional helper virus and/or a packaging cell line. Homologous recombination occurs between the helper and the vector, which permits the adenovirus-transgene sequences in the vector to be replicated and packaged into virion capsids, resulting in the recombinant vector viral particles. The current method for producing such virus particles is transfection-based. Briefly, helper virus is used to infect cells, such as the packaging cell line human HEK 293, which are then subsequently transfected with an adenovirus plasmid vector containing a VLDLR transgene by conventional methods. About 30 or more hours post-transfection, the cells are harvested, an extract

prepared and the recombinant virus vector containing the VLDLR transgene is purified by buoyant density ultracentrifugation in a CsCl gradient.

The yield of transducing viral

5 particles is largely dependent on the number of cells that are transfected with the plasmid, making it desirable to use a transfection protocol with high efficiency. One such method involves use of a poly-L-lysinylated helper adenovirus as described above. A
10 plasmid containing the VLDLR minigene is then complexed directly to the positively charged helper virus capsid, resulting in the formation of a single transfection particle containing the plasmid vector and the helper functions of the helper virus.

15 *II. Use of the Recombinant Virus Vectors in Gene Therapy*

The resulting recombinant adenoviral vector containing the VLDLR minigene produced by cooperation of the adenovirus vector and helper virus or adenoviral vector and packaging cell line, as described above, thus
20 provides an efficient gene transfer vehicle which can deliver the VLDLR gene to a patient *in vivo* or *ex vivo* and provide for integration of the gene into a liver cell.

The above-described recombinant vectors are
25 administered to humans in a conventional manner for gene therapy and serve as an alternative or supplemental gene therapy for LDL receptor deficiencies or other lipoprotein metabolic disorders. A viral vector bearing the VLDLR gene may be administered to a patient,
30 preferably suspended in a biologically compatible solution or pharmaceutically acceptable delivery vehicle. A suitable vehicle includes sterile saline. Other aqueous and non-aqueous isotonic sterile injection solutions and aqueous and non-aqueous sterile suspensions

known to be pharmaceutically acceptable carriers and well known to those of skill in the art may be employed for this purpose.

The viral vectors are administered in sufficient amounts to transfect the liver cells and provide sufficient levels of transfer and expression of the VLDLR gene to provide a therapeutic benefit without undue adverse or with medically acceptable physiological effects which can be determined by those skilled in the medical arts. Conventional and pharmaceutically acceptable routes of administration include direct delivery to the liver, intranasal, intravenous, intramuscular, subcutaneous, intradermal, oral and other parental routes of administration. Routes of administration may be combined, if desired.

Dosages of the viral vector will depend primarily on factors such as the condition being treated, the age, weight and health of the patient, and may thus vary among patients. For example, a therapeutically effective human dosage of the viral vector is generally in the range of from about 20 to about 100 ml of saline solution containing concentrations of from about 1×10^9 to 1×10^{11} pfu/ml virus vector. A preferred human dosage is estimated to be about 50 ml saline solution at 2×10^{10} pfu/ml. The dosage will be adjusted to balance the therapeutic benefit against any adverse side effects. The levels of expression of the VLDLR gene can be monitored to determine the frequency of dosage administration.

An optional method step involves the co-administration to the patient, either concurrently with, or before or after administration of the viral vector, of a suitable amount of an immune modulator, which is preferably short-acting. The selected immune modulator is defined herein as an agent capable of inhibiting the

formation of neutralizing antibodies directed against products of the recombinant vector of this invention and/or capable of inhibiting cytolytic T lymphocyte (CTL) elimination of the vector containing cells. The immune modulator may interfere with the interactions between the T helper subsets (T_{H1} or T_{H2}) and B cells to inhibit neutralizing antibody formation. Alternatively, the immune modulator may be selected to inhibit the interaction between T_{H1} cells and CTLs to reduce the occurrence of CTL elimination of the vector. More specifically, the immune modulator desirably interferes with, or blocks, the function of the CD4 T cells.

Immune modulators for use in inhibiting neutralizing antibody formation may be selected based on the determination of the immunoglobulin subtype of any neutralizing antibody produced in response to the VLDLR-containing adenovirus vector. For example, if the neutralizing antibody is a T_{H2} mediated antibody, such as IgA, the immune modulator desirably suppresses or prevents the interaction of T_{H2} with B cells. Alternatively, if the induced neutralizing antibody is a T_{H1} mediated antibody, such as IgG_{2A}, the immune modulator desirably suppresses or prevents the interaction of T_{H1} with B cells.

The neutralizing antibody which develops in response to administration of a viral vector of this invention can be based on what vehicle is being used to deliver the vector and/or the location of delivery. For instance, administration of adenoviral vectors via the lungs generally induces production of IgA neutralizing antibody. Administration of adenoviral vectors via the blood generally induces IgG, neutralizing antibody. The determination of the neutralizing antibody is readily determined in trials of the selected viral vector in animal models. Where the reduction of CTL elimination of

the viral vectors is desired, the immun modulator is selected for its ability to suppress or block CD4⁺ T_{H1} cells to permit prolonged residence of the viral vector in vitro.

5 Selection of the immune modulator thus may be based upon the mechanism sought to be interrupted or blocked. The immune modulators may be soluble proteins or naturally occurring proteins, including cytokines, monoclonal antibodies. The immune modulators may be conventional pharmaceuticals. The immune modulators identified herein may be used alone or in combination with one another. For example, cyclophosphamide and the more specific immune modulator anti-CD4 monoclonal antibody may be co-administered. In such a case, 10 cyclophosphamide serves as an agent to block T_{H1} activation and stabilized transgene expression beyond the period of transient immune blockade.

15

A suitable amount or dosage of the immune modulator will depend primarily on the amount of the recombinant vector bearing the VLDLR gene which is initially administered to the patient and the type of 20 immune modulator selected. Other secondary factors such as the condition being treated, the age, weight, general health, and immune status of the patient, may also be considered by a physician in determining the dosage of 25 immune modulator to be delivered to the patient.

Generally, for example, a therapeutically effective human dosage of a cytokine immune modulator, e.g., IL-12 or γ -IFN, is generally in the range of from 30 about 0.5 μ g to about 5 mg per about 1 x 10⁷ pfu/ml virus vector. Various dosages may be determined by one of skill in the art to balance the therapeutic benefit against any side effects.

A. *Monoclonal Antibodies and Soluble Proteins*

Preferably, the method of inhibiting an adverse immune response to the gene therapy vector involves non-specific inactivation of CD4⁺ cells.

5 Preferably, such blocking antibodies are "humanized" to prevent the recipient from mounting an immune response to the blocking antibody. A "humanized antibody" refers to an antibody having its complementarily determining regions (CDRs) and/or other portions of its light and/or

10 heavy variable domain framework regions derived from a non-human donor immunoglobulin, the remaining immunoglobulin-derived parts of the molecule being derived from one or more human immunoglobulins. Such antibodies can also include antibodies characterized by a

15 humanized heavy chain associated with a donor or acceptor unmodified light chain or a chimeric light chain, or vice versa. Such "humanization" may be accomplished by methods known to the art. See, for example, G.E. Mark and E. A. Padlan, "Chap. 4. Humanization of Monoclonal

20 Antibodies", The Handbook of Experimental Pharmacology, vol. 113, Springer-Verlag, New York (1994), pp. 105-133, which is incorporated by reference herein.

Other suitable antibodies include those that specifically inhibit or deplete CD4⁺ cells, such as an antibody directed against cell surface CD4. Depletion of CD4⁺ cells has been shown by the inventors to inhibit the CTL elimination of the viral vector. Such modulatory agents include but are not limited to anti-T cell antibodies, such as anti-OKT3+ [see, e.g., US Patent No. 4,658,019; European Patent Application No. 501,233, published September 2, 1992]. See Example 2 below, which employs the commercially available antibody GK1.5 (ATCC Accession No. TIB207) to deplete CD4⁺ cells.

Alternatively, any agent that interferes with or blocks the interactions necessary for the activation of B cells by T_H cells, and thus the production of neutralizing antibodies, is useful as an immune modulator according to these methods. For example, B cell activation by T cells requires certain interactions to occur [F. H. Durie et al, Immunol. Today, 15(9):406-410 (1994)], such as the binding of CD40 ligand on the T helper cell to the CD40 antigen on the B cell, and the binding of the CD28 and/or CTLA4 ligands on the T cell to the B7 antigen on the B cell. Without both interactions, the B cell cannot be activated to induce production of the neutralizing antibody.

The CD40 ligand (CD40L)-CD40 interaction is a desirable point to block the immune response to gene therapy vectors because of its broad activity in both T helper cell activation and function as well as the absence of redundancy in its signaling pathway. A currently preferred method of the present invention thus involves transiently blocking the interaction of CD40L with CD40 at the time of adenoviral vector administration. This can be accomplished by treating with an agent which blocks the CD40 ligand on the T_H cell and interferes with the normal binding of CD40 ligand on the T helper cell with the CD40 antigen on the B cell. Blocking CD40L-CD40 interaction prevents the activation of the T helper cells that contributes to problems with transgene stability and readministration.

Thus, an antibody to CD40 ligand (anti-CD40L) [available from Bristol-Myers Squibb Co; see, e.g., European patent application 555,880, published August 18, 1993] or a soluble CD40 molecule can be a selected immune modulator in this method.

Alternatively, an agent which blocks the CD28 and/or CTLA4 ligands present on T helper cells interferes with the normal binding of those ligands with the antigen B7 on the B cell. Thus, a soluble form of B7 or an antibody to CD28 or CTLA4, e.g., CTLA4-Ig [available from Bristol-Myers Squibb Co; see, e.g., European patent application 606,217, published July 20, 1994] can be the selected immune modulator in the method of this invention. This method has greater advantages than the below-described cytokine administration to prevent T_{H2} activation, because it addresses both cellular and humoral immune responses to foreign antigens.

B. Cytokines

Still other immune modulators which inhibit the T_H cell function may be employed in this invention.

Thus, in one embodiment, an immune modulator which selectively inhibits the function of the T_{H1} subset of CD4 $^+$ T helper cells may be administered at the time of primary administration of the viral vector. One such immune modulator is interleukin-4 (IL-4). IL-4 enhances antigen specific activity of T_{H2} cells at the expense of the T_{H1} cell function [see, e.g., Yokota et al, *Proc. Natl. Acad. Sci., USA*, **83**:5894-5898 (1986); United States Patent No. 5,017,691]. It is envisioned that other immune modulators that can inhibit T_{H1} cell function will also be useful in the methods of this invention.

In another embodiment, the immune modulator can be a cytokine that prevents the activation of the T_{H2} subset of T helper cells. The success of this method depends on the relative contribution that T_{H2} dependent Ig isotypes play in virus neutralization, the profile of which may be affected by strain, the species

of animal as well as the mode of virus delivery and target organ.

A desirable immune modulator which selectively inhibits the CD4⁺ T cell subset T_{H2} function at the time of primary administration of the viral vector includes interleukin-12 (IL-12). IL-12 enhances antigen specific activity of T_{H1} cells at the expense of T_{H2} cell function [see, e.g., European Patent Application No. 441,900; P. Scott, Science, 260:496-497 (1993); R. Manetti et al, J. Exp. Med., 177:1199 (1993); A. D'Andrea et al, J. Exp. Med., 176:1387 (1992)]. IL-12 for use in this method is preferably in protein form. Human IL-12 may be recombinantly produced using known techniques or may be obtained commercially. Alternatively, it may be engineered into a viral vector (which optionally may be the same as that used to express the transgene) and expressed in a target cell *in vivo* or *ex vivo*.

T_{H2} specific ablation with IL-12 is particularly effective in lung-directed gene therapies where IgA is the primary source of neutralizing antibody. In liver-directed gene therapy, both T_{H1} and T_{H2} cells contribute to the production of virus specific antibodies. However, the total amount of neutralizing antibody can be diminished with IL-12.

Another selected immune modulator which performs a similar function is gamma interferon (IFN- γ) [S. C. Morris et al, J. Immunol., 152:1047-1056 (1994); F. P. Heinzel et al, J. Exp. Med., 177:1505 (1993)]. IFN- γ is believed to mediate many of the biological effects of IL-12 via secretion of activated macrophages and T helper cells. IFN- γ also partially inhibits IL-4 stimulated activation of T_{H2}. IFN- γ may also be obtained from a variety of commercial sources.

Alternatively, it may be engineered into a viral vector and expressed in a target cell *in vivo* or *ex vivo* using known genetic engineering techniques.

Preferably, such cytokine immune modulators are in the form of human recombinant proteins. These proteins may be produced by methods extant in the art. Active peptides, fragments, subunits or analogs of the known immune modulators described herein, such as IL-12 or gamma interferon, which share the T_{H2} inhibitory function of these proteins, will also be useful in this method when the neutralizing antibodies are T_{H2} mediated.

C. Other Pharmaceuticals

Other immune modulators or agents that non-specifically inhibit immune function, i.e., cyclosporin A or cyclophosphamide, may also be used in the methods of the invention. For example, a short course of cyclophosphamide has been demonstrated to successfully interrupt both CD4 and CD8 T helper cell activation to adenovirus capsid protein at the time of virus delivery to the liver. As a result, transgene expression was prolonged and, at higher doses, formation of neutralizing antibody was prevented, allowing successful vector readministration. In the lung, cyclophosphamide prevented formation of neutralizing antibodies at all doses and stabilized transgene expression at high dose.

D. Administration of Immune Modulator

The optional administration of the selected immune modulator may be repeated during the treatment with the recombinant adenovirus vector carrying the human VLDLR gene, during the period of time that the VLDLR gene is expressed (as monitored by e.g., LDL levels), or with every booster of the recombinant vector.

Thus, the compositions and methods of this invention provide a desirable treatment for defects in *LDL* metabolism, by providing stable expression of the *VLDLR* gene in human hepatocytes, and the ability to 5 administer the vector as desired without incurring an undesired immune response by the patient.

The following examples illustrate the construction and testing of the viral vectors and *VLDL* receptor gene inserts of the present invention and the 10 use thereof in the treatment of metabolic disorders. An exemplary recombinant adenovirus encoding the human *VLDL* receptor was constructed as described in Example 1 below. These examples are illustrative only, and do not limit the scope of the present invention.

15 Example 1 - Construction and Purification of H5.010CMVVLDLR

The cDNA for the human very low density lipoprotein (*VLDL*) receptor [M. E. Gafvels et al, cited above; SEQ ID NO: 1] was inserted into the polylinker 20 region of plasmid pRc/CMV (obtained from Invitrogen Corp.). The resulting plasmid, pRc/CMVVLDLR, was digested with the restriction enzymes SnaBI and NotI and the 4 kb fragment containing the cytomegalovirus (CMV) immediate-early promoter and *VLDL* receptor cDNA was 25 isolated.

The plasmid pAd.CMVlacZ [Kozarsky II, cited above] was digested with SnaBI and NotI to remove the CMV promoter and lacZ cDNA and the 5.6 kb backbone was isolated. The two fragments were ligated to generate 30 pAd.CMVVLDLR (Figs. 2 and 9; SEQ ID NO: 3). pAd.CMVVLDLR was linearized with NheI and co-transfected into 293 cells with sub360 DNA (derived from adenovirus type 5) which had been digested with XbaI and ClaI as previously described [K. F. Kozarsky I and II cited above].

The resulting recombinant adenovirus, designated H5.010CMVVLDLR, contains the sequence from about nucleotide 12 to about 4390 of pAd.CMVVL_DLR and Ad.5 map units 9-100 with a small deletion in the E3 gene (see GenBank Accession No. M73260 and discussion of Fig. 3). This recombinant adenovirus was isolated following two rounds of plaque purification. H5.010CMVVLDLR was grown on 293 cells and purified by two rounds of cesium chloride density centrifugation as previously described [K. F. Kozarsky I and II cited above]. Cesium chloride was removed by passing the virus over a BioRad 10DG desalting column equilibrated with phosphate-buffered saline.

For rabbit experiments, virus was used freshly purified; for mouse experiments, virus was either used fresh, or after column purification glycerol was added to a final concentration of 10% (v/v), and virus was stored at -70°C until use.

As described in the following examples, this recombinant adenovirus vector was introduced into the livers of WHHL rabbits and into the livers of LDL receptor knockout mice to determine the *in vivo* function of the VLDL receptor, and to determine its usefulness as an alternative or supplemental gene therapy for LDL receptor deficiency.

Example 2 - Other Recombinant Adenoviruses

H5.010CMVlacZ, encoding the lacZ gene under the control of the CMV enhancer/promoter, and H5.010CBhLDLR, encoding the human low density lipoprotein (LDL) receptor cDNA under the control of the CMV-enhanced chicken β -actin promoter, were prepared as previously described [K. F. Kozarsky I and II, cited above].

Example 3 - Effects of Hepatic Expression of the VLDL Receptor in the WHHL Rabbit

H5.010CMVVLDLR or H5.010CMVlacZ (encoding β -galactosidase), obtained as described in Examples 1 and 2, was infused intravenously into WHHL rabbits [Camm Research] as follows. Rabbits were infused with 7.5 x 10¹² particles of either recombinant adenovirus through a marginal ear vein on day 0. In addition, two New Zealand White (NZW) rabbits [Hazleton, Inc.] were infused with each virus and sacrificed on day 5 post-infusion to document the extent of gene transfer in the liver.

Rabbits were maintained in a 12 hour light/dark cycle on a diet of Purina laboratory chow, delivered each day at approximately 11:00 am. Venous samples were obtained through a marginal ear vein at approximately 10:00 am on the days indicated.

A. Plasma Analyses

Plasma samples were analyzed for total cholesterol using the Cholesterol HP kit and Precise standards (Boehringer Mannheim). Briefly, FPLC analysis was performed on 50 μ l of plasma from individual mice adjusted to a volume of 250 μ l in FPLC column buffer (1 mM EDTA, 154 mM NaCl, pH 8.0). Diluted samples (200 μ l) were loaded onto two Superose 6 columns (Pharmacia) in series at a flow rate of 0.4 ml/min, and 1 ml fractions were collected. Cholesterol content was analyzed in a microplate assay on 100 μ l samples. 100 μ l of a freshly prepared solution containing 50 mM PIPES, pH 6.9, 7.8 g/L HDCBS, 0.51 g/L 4-AAT, 1.27 g/L cholic acid, 0.245% Triton X-100, 7.31 g/L KCl and supplemented with 1.22 U/ml cholesterol oxidase, 7.64 U/ml cholesterol esterase, and 245 U/ml peroxidase was added to samples, incubated overnight at room temperature, and the O.D. at 490 nm was determined.

Plasma cholesterol levels were evaluated in each of the WHHL rabbits before and after receiving recombinant adenovirus. Fig. 4A shows that rabbits infused with H5.010CMVlacZ had no significant changes in cholesterol levels. However, following infusion with H5.010CMVVLDLR, cholesterol levels dropped, with maximum decreases that ranged from 140 to 420 mg/dl (Fig. 4B). This demonstrated that expression of the VLDL receptor results in decreased cholesterol levels in LDL receptor-deficient rabbits.

B. Histochemical Analysis

Portions of liver were paraffin embedded, sectioned, and stained with hematoxylin and eosin. Some portions were fresh-frozen, sectioned, fixed in glutaraldehyde, stained with X-gal and lightly counterstained with hematoxylin. Some fresh-frozen sections were fixed in methanol, and then stained with either a polyclonal anti- β -galactosidase antibody (5 prime-3 prime), a polyclonal anti-human LDL receptor antibody, or with a polyclonal anti-VLDL receptor antibody, followed by a fluorescein isothiocyanate-conjugated anti-rabbit antibody (Jackson Immunoresearch) as previously described [K. F. Kozarsky I and II cited above]. Oil Red O staining was performed on fresh-frozen sections fixed for 1 minute in 37% formaldehyde, then rinsed and stained in Oil Red O (3 parts 0.5% Oil Red O in isopropyl alcohol/2 parts water) for 10 minutes. Slides were counterstained in hematoxylin and mounted in aqueous solution.

Immunofluorescence analysis of the infused rabbits showed that approximately 50% of hepatocytes from the rabbit infused with H5.010CMVlacZ expressed β -galactosidase, liver tissue from the rabbit infused with H5.010CMVVLDLR had a slightly higher percentage of hepatocytes expressing the VLDL receptor. In agreement

with Northern blot analysis showing little or no VLDL receptor mRNA expression [M. E. Gafvels et al, cited above], liver from the lacZ-infused rabbit showed no reactivity with the anti-VLDL receptor antibody.

5 Example 4 - Effects of Short-Term Hepatic Expression of
the VLDL Receptor in LDL Receptor Knockout Mice

C57BL/6 mice and LDL receptor knockout mice (Jackson Labs) were infused intravenously with 0.5 or 1.0 x 10¹⁰ particles of recombinant adenovirus through the 10 tail vein and cholesterol levels were monitored before and after infusion.

Specifically, three mice each were infused with either H5.010CMVlacZ, H5.010CMVVLDLR, or H5.010CBhLDLR (encoding the human LDL receptor cDNA). This last virus 15 was included as a control to confirm published results [Kozarsky I and II cited above]. Plasma samples were obtained by retro-orbital bleeds using heparinized capillary tubes. The LDL receptor knockout mice were maintained upon a high cholesterol diet composed of 20 Purina mouse chow supplemented with 1.25% cholesterol, 7.5% cocoa butter, 7.5% casein, and 0.5% cholate (1.25% cholesterol diet) for at least 3 weeks immediately following weaning before experiments were initiated. Mice were sacrificed on day 5 post-infusion.

25 Liver tissues were analyzed by immunofluorescence for transgene expression by the techniques described in Example 3, and plasma cholesterol levels were measured as similarly described. For 30 lipoprotein fractionations, plasma from triplicate LDL receptor knockout mice were pooled, subjected to density ultracentrifugation, fractions were collected, and the cholesterol content was determined by conventional means.

Immunofluorescence analysis revealed moderate levels of β -galactosidase expression in H5.010CMVlacZ-infused mice, and higher levels of either human *LDL* receptor and VLDL receptor expression in H5.010CBhLDLR-5 and in H5.010CMVVLDLR-infused mice, respectively.

Cholesterol levels decreased slightly in the control, H5.010CMVlacZ-infused mice (Fig. 5), probably due to non-transgene-related effects of infusion of recombinant adenovirus, which can result in 10 hepatotoxicity in mice [Y. Yang et al, *Proc. Natl. Acad. Sci., USA*, 91:4407-4411 (May 1994)]. However, in contrast to the decrease observed in the control mice, cholesterol levels dropped significantly to 50% of pre-infusion values in the H5.010CBhLDLR-infused mice on day 15 5 post-infusion. Cholesterol levels in the H5.010CMVVLDLR-infused mice also decreased, to approximately 60% of pre-infusion levels. Further analysis of plasma lipoproteins showed that in the H5.010CBhLDLR-treated mice, *LDL* levels plummeted, with 20 additional decreases in *IDL* and *VLDL* fractions (Fig. 6). The H5.010CMVVLDLR-infused mice showed a larger decrease in the *VLDL* fraction with less of a decrease in *LDL*.

Taken together, these data indicate that 25 hepatic expression of *VLDL* receptor results in increased clearance of *VLDL* from the plasma, resulting in decreases in the amounts of lipoproteins for which *VLDL* is the precursor (i.e., *IDL* and *LDL*), and an overall drop in total plasma cholesterol.

Example 5 - Effects of Long-Term Hepatic Expression of 30 the *VLDL* Receptor in *LDL* Receptor Knockout Mice

In order to achieve cholesterol levels closer to those observed in both FH patients and WHHL rabbits, *LDL* receptor knockout mice (Jackson Labs) were maintained on a high cholesterol diet composed of Purina mouse chow

supplemented with 0.2% cholesterol, 10% coconut oil, and 0.05% cholate (0.2% cholesterol diet). Cholesterol levels in these mice ranged from 930 to 1550 mg/dl, whereas the mice on the 1.25% cholesterol (Example 4) 5 diet had levels of 1900 to 3100 mg/dl.

Virus was thawed immediately before use and diluted with PBS to a concentration of 1×10^{12} particles/ml. Three mice were each infused intravenously with 0.1 ml of virus containing 1×10^{11} particles of an 10 E1-deleted recombinant adenovirus encoding either β -galactosidase (H5.010CMVlacZ) or human LDL receptor (H5.010CBhLDLR), and serum lipids were followed over time. On the days indicated, mice were anesthetized with methoxyflurane and blood was collected into heparinized 15 capillary tubes by puncture of the retro-orbital venous plexus.

Immunofluorescence staining showed that most of the hepatocytes expressed the transgene product, either β -galactosidase, human LDL receptor, or VLDL receptor. 20 Hematoxylin and eosin staining of sections of liver revealed essentially normal morphology in the H5.010CMVlacZ-infused mouse. However, for both the H5.010CBhLDLR- and H5.010CMVVLDLR-infused mice, hepatocytes appeared to have internal vacuoles. When 25 tissue was analyzed with Oil Red O staining, a stain for neutral lipids, liver from the receptor-infused animals clearly showed accumulation of large droplets of lipid when compared with the H5.010CMVlacZ-infused control. This suggested that short-term, high level expression of 30 the LDL receptor or VLDL receptor in these LDL receptor-deficient mice resulted in intracellular accumulation of lipids.

To confirm the biological activities of the transgene products, plasma cholesterol levels were 35 followed before and after recombinant adenovirus

administration. Fig. 7A shows that serum chol sterol levels in H5.010CMVlacZ-infused mice demonstrated a characteristic but not significant fluctuation over time, reflected in minor changes of all lipoprotein fractions (HDL, IDL/VLDL, and LDL). In contrast, mice infused with H5.010CBhLDLR have a large but transient decrease in cholesterol (see, Fig. 7B). Particularly, these mice demonstrated large plasma cholesterol decreases which lasted for approximately 2 weeks. Cholesterol levels decreased 3-fold (from 966 to 353 mg/dl) and 7-fold (from 1554 to 219 mg/dl) and returned to baseline by 3 weeks post-infusion. The decrease in serum cholesterol is reflected in coordinate diminution in serum LDL. This nonspecific effect of the adenovirus infection when immune modulators are not coordinately administered has been described previously and is likely due to changes in hepatic function that occur as a result of the associated inflammation. Mice infused with H5.010CMVLDLR showed large decreases in plasma cholesterol which were similar in magnitude to those seen in the H5.010CBhLDLR-infused mice (Fig. 7C), with maximum decreases of more than 4-fold (from 1186 to 288 mg/dl and from 1453 to 299 mg/dl). Surprisingly, plasma cholesterol levels did not return to baseline by 3 weeks post-infusion. The change in plasma cholesterol levels in the H5.010CMVLDLR-infused mice (Fig. 7A) were statistically significant ($p<0.05$) through 9 weeks following infusion (the current duration of the experiment).

Sera from individual mice was analyzed by FPLC to determine the effects of VLDL receptor expression on lipoprotein fractions. On day 3 post-infusion, VLDL and LDL fractions were undetectable; over time, the LDL fraction slowly recovered, although even at 10 weeks post-infusion, the LDL peak height was slightly lower than the HDL peak height. VLDL remained undetectable

although minor differences may escape detection because of limitations in the sensitivity of the cholesterol assay. The LDL peaks mirrored the total plasma cholesterol levels, and confirmed that the prolonged 5 lowering of plasma cholesterol was accompanied by sustained decreases in VLDL and LDL levels. These data suggest that expression of the VLDL receptor in the liver is an effective therapy for hypercholesterolemia.

At the same time of infusion of the LDL receptor knockout mice, normal C57Bl/6 mice were infused 10 with each of the recombinant adenoviruses. These mice were sacrificed at various times post-infusion, and liver tissues were harvested for direct analysis of transgene expression using X-gal histochemistry to detect β -galactosidase expression and immunofluorescence performed 15 to measure LDL receptor expression. Tissues harvested three days after infusion of virus demonstrated either expression of β -galactosidase or the human LDL receptor in at least 80% of hepatocytes.

In each experiment, the vector specific signal 20 was substantially higher than that seen in animals before gene transfer or following infusion with identical quantities of an adenovirus expressing an irrelevant gene. For both lacZ and LDL receptor, transgene 25 expression diminished to undetectable levels by day 21 and was associated with the development of a self limited mononuclear infiltrate in liver that peaked at day 10. The infiltrate consisted of portal as well as lobular inflammation, accompanied by the presence of apoptic 30 bodies. The extent of pathology was indistinguishable between the lacZ and LDL receptor infused mice. The time course of LDL receptor expression is consistent with the initial large decline in plasma cholesterol and subsequent return to baseline.

In contrast, two mice infused with H5.010CMVVLDLR expressed the VLDL receptor at high levels. The percent of hepatocytes may have decreased slightly as compared to the day 5 mice. These data 5 suggest that the sustained decrease in plasma cholesterol levels in the H5.010CMVVLDLR-infused mice was due to sustained expression of the VLDL receptor.

Example 6 - Turnover Studies

To further characterize the effects of hepatic 10 VLDL receptor expression on lipoprotein metabolism, turnover studies were performed as follows.

LDL receptor knockout mice were infused with recombinant adenovirus after 3 weeks on the high cholesterol diet as described in Example 4. Three mice 15 each were injected with the lacZ and VLDL receptor adenoviruses; one mouse was injected with the LDL receptor adenovirus. On day 5 post-infusion, mice were injected via the tail vein with approximately 8×10^6 cpm of ^{125}I -labeled human LDL, and 1.6×10^5 cpm of ^{131}I -labeled 20 human VLDL in a total volume of 0.2 ml. A blood sample was obtained 1 minute following injection of radiolabel, and designated the "time zero" sample. Blood was collected into heparinized capillary tubes at the indicated times, and radioactivity remaining was 25 determined using a gamma counter.

Infusion of LDL receptor adenovirus led to accelerated clearance of LDL as compared to infusion of lacZ adenovirus, consistent with a previous study in LDL receptor knockout mice [S. Ishibashi et al, J. Clin. Invest., 92:883-893(1993)]. Similarly, VLDL clearance 30 was accelerated in LDL receptor treated animals as compared to lacZ infused mice. LDL turnover in VLDL receptor-infused mice was indistinguishable from lacZ infused mice, consistent with *in vitro* data which

indicates that LDL is not a ligand for the VLDL receptor [T. Yamamoto et al, *Trends in Cardiovascular Medicine*, 3:144-148 (1993); F. Batley et al, *J. Biol. Chem.*, 269:23268-23273 (1994)]. VLDL clearance in VLDL receptor infused mice was slightly faster than in lacZ infused mice, but significantly slower than in LDL receptor infused mice.

As discussed above, VLDL turnover in mice infused with the VLDL receptor adenovirus was significantly faster than in lacZ infused mice although the magnitude of this effect was far less than that seen in animals treated with LDL receptor virus. This suggests that VLDL receptor-mediated clearance of circulating VLDL may not be the only pathway leading to diminished serum VLDL. One potential mechanism is secretion-recapture, which occurs with hepatic uptake of chylomicron remnants [T. Willnow & J. Herz, *J. Mol. Med.*, 73:213-220 (1995); H. Shimano et al, *J. Clin. Invest.*, 93:2215-2223 (1994)], and would result in decreased secretion of VLDL and reduced levels of plasma VLDL. A second mechanism may involve the interaction of the VLDL receptor with receptor-associated protein (RAP) [Battey, cited above; H. Mokuno et al, *J. Biol. Chem.*, 269:13238-13243 (1994)] which interacts with a variety of receptors inside the cell, apparently to prevent ligand binding before the receptor reaches the cell surface [G. Bu et al, *EMBO J.*, 14:2269-2280 (1995)]. It is possible that the high levels of VLDL receptor expressed in the livers of adenovirus-infused mice overwhelms the available RAP, so that VLDL receptor is binding to newly synthesized ligand (apoE, either free or in association with lipid) within the cell, and preventing its secretion into the plasma. The effects of hepatic VLDL receptor expression on total plasma cholesterol as well as on lipoprotein

cholesterol levels demonstrate that the VLDL receptor can play a major role in lipoprotein metabolism in vivo.

Example 7 - Stability of Expression of VLDL Receptor

This experiment illustrates relative transgene
5 persistence in mice.

LDL receptor knockout mice were injected intravenously on day 0 with 1×10^{11} particles of H5.010CMVlacZ, H5.010CBhLDLR, or H5.010CMVVLDLR. Mice were sacrificed on the indicated days after injection (3,
10 10 or 21), and fresh-frozen sections of liver were stained with X-gal to detect expression of the lacZ gene, and with anti-LDL receptor antibody or anti-VLDL receptor antibody, followed by a fluorescein-conjugated secondary antibody to detect LDL receptor and VLDL receptor,
15 respectively.

Analysis of liver harvested 3 days after infusion of virus revealed VLDL receptor protein in >80% of hepatocytes; the bright fluorescent signal, which localized to the perimeter of the cell, was absent before gene transfer and in tissues of animals infected with lacZ or LDL receptor containing adenoviruses. Expression of VLDL receptor protein was remarkably stable with recombinant protein detected in approximately 5 to 10% of hepatocytes from tissue harvested 105 days after infusion of virus. This is in striking contrast to the results obtained with lacZ and LDL receptor adenovirus, where expression of the transgene extinguished to undetectable levels within three weeks of gene transfer. VLDL receptor expression remained detectable through the duration of the experiment (22 weeks).

Genomic DNA was isolated from mouse liver, digested with EcoRI, and subjected to Southern blotting [K. Kozarsky et al. J. Biol. Chem., 269:13695-13702 (1994)] to monitor the presence over time of adenoviral

DNA sequences. Adenovirus sequences were detected using the Genius kit from Boehringer Mannheim, followed by chemiluminescent detection. In C57BL/6 mice infused with the lacZ adenovirus, viral DNA diminished rapidly with time, plateauing at barely detectable levels (~0.05 copies/cell) through day 70 post-infusion. Mice infused with VLDL receptor had slightly higher initial levels of DNA, but a similar time course of loss of adenovirus sequences. Additional DNA hybridization studies showed that the majority of adenovirus DNA initially delivered to the liver is not integrated into the mouse genome (data not shown), however, this assay cannot rule out some level of integration.

Histopathologic analysis of liver tissue from mice infused with the VLDL receptor virus revealed inflammation and apoptotic cells at early time points. The timing and extent of the pathologic findings were indistinguishable from liver tissues of mice infused with lacZ and LDL receptor viruses. At 15 and 22 weeks post-infusion, however, liver tissue from VLDL receptor-infused mice displayed discernible accumulations of neutral lipids, as demonstrated by hematoxylin and eosin as well as oil red O staining. Similar changes were observed infrequently in LDL receptor knockout mice infused with PBS, LDL receptor and/or lacZ adenoviruses. No lipid accumulations were observed in livers of normal C57BL/6 mice infused with the VLDL receptor virus, despite long-term transgene expression indistinguishable from that observed in LDL receptor knockout mice. This indicates that VLDL receptor expression alone is not sufficient for the changes in lipid accumulation observed in LDL receptor knockout mice; instead, there is some lipid accumulation in the LDL receptor knockout mice which have been maintained on a high cholesterol diet for

≥ 18 weeks, that is accelerated by prolonged VLDL receptor expression.

Plasma samples from mice infused with VLDL receptor adenovirus were analyzed for the presence of 5 antibodies directed against the VLDL receptor protein. Only one mouse out of twelve generated antibodies to the VLDL receptor despite the presence of high level antibodies to adenovirus capsid proteins in each animal that received virus. Animals infused with the VLDL 10 receptor adenovirus mounted a CTL response to adenoviral proteins indistinguishable from that obtained from animals infused with either lacZ or LDL receptor adenoviruses. These mice, however, did not mount a CTL response to the VLDL receptor protein. Thus, the 15 development of a CTL response to the transgene following infusion of recombinant adenovirus is dependent on the antigenicity of the specific transgene in the treated animal.

Example 8 - Humoral and Cellular Immune Response to
20 Adenovirus and Transgenes

A. Humoral Immune Response

Two LDL receptor knockout mice (K020 and K027) or two normal C57BL/6 mice were injected via the tail vein with 1×10^{11} particles of H5.010CBhLDLR at day 0 25 and serum samples were collected both before injection (pre), and on days 10, 24, 39, 52 and 70 following injection for the knockout mice and on day 21 for the C57BL/6 mice. Western blots were performed as previously described [K. Kozarsky et al, J. Biol. Chem., 269:13695-30 13702 (1994); K. Kozarsky et al., Som. Cell and Molec. Genet., 19:449-458 (1993)]. To detect anti-adenovirus antibodies, purified adenovirus was used as the antigen.

The positive control (+) was rabbit antiserum isolated following intravenous infusion of purified H5.010CBhLDLR. The negative control (-) was pre-immune rabbit serum. Western blots with β -galactosidase were performed using 5 purified protein (Sigma), with a monoclonal antibody specific for β -galactosidase (Sigma) as a positive control.

Antibodies directed against the human LDL receptor were detected using lysates prepared from 24-23 10 cells, a 3T3 cell line which was transduced with retrovirus encoding the human LDL receptor. For detection of anti-VLDL receptor antibodies, a lysate was prepared from HeLa cells two days following infection with H5.010 CMVVLDLR.

15 All mice infused with 1×10^{11} particles of recombinant adenovirus developed antibodies to adenovirus capsid proteins, with major bands corresponding to hexon, penton and fiber. All mice infused with H5.010CBhLDLR developed antibodies to the human LDL receptor protein 20 with LDL receptor knockout mice consistently developing higher titer antibodies than C57BL/6 mice. Antibodies from LDL receptor knockout mice cross-reacted with mouse LDL receptor protein, whereas antibodies from C57BL/6 mice (which express normal mouse LDL receptor) did not.

25 This suggests that the VLDL receptor, although the human and not the mouse sequence was used, was not immunogenic in these mice. The amino acid sequences of the human and mouse LDL receptors are approximately 78% identical, while the human and mouse VLDL receptors are >94% identical. This increased sequence similarity is likely to account for the absence 30 of antibody development to the human VLDL receptor despite high level expression in the mouse liver as a result of infusion of H5.010CMVVLDLR.

The data demonstrate that animals can generate a humoral immune response specific for the transgene product as well as to the viral proteins encoded on the injected adenovirus. It also provides
5 indirect evidence of antigen specific activation of T helper cells, which is normally required for development of mature, antibody-secreting B cells.

B. Cellular Immune Responses

This study analyzed animals following
10 infusion with the LDL receptor adenovirus for activation of CTLs to both viral antigens and the transgene product, human LDL receptor.

CTL assays were performed as described in Y. Yang et al, Immunity, 1:433-442 (1994). Target cells
15 expressing recombinant vaccinia proteins were generated by infecting with recombinant vaccinia were generated as follows. The VLDV receptor cDNA (in the pRC/CMV plasmid) was subcloned into the HindIII site of Bluescript KS+. The CFTR cDNA [J.R. Riordan et al, Science, 245:1066-1073
20 (1989) was cloned into the PstI site of Bluescript KS+ (Stratagene). The LDL receptor cDNA in the pUC19 vector [T. Yamamoto et al, Cell, 39:27-38 (1984)] was excised with the restriction enzymes HindIII and Sac I and ligated into the HindIII and SacI sites of Bluescript
25 KS+. Each of the cDNAs was then excised using the enzymes SacII and KpnI and cloned into the SacII and KpnI sites of a modified form of the vaccinia expression vector pSC11 [S. Chakrabarti et al, Molec. Cell. Biol., 5:3403-3409 (1985)]. The control recombinant vaccinia,
30 VRG, expresses a rabies virus glycoprotein and was prepared as described in T. Wiktor et al, Proc. Natl. Acad. Sci. USA, 81: 7194-7198 (1984).

35 CTLs to specific targets were detected in a standard ⁵¹chromium (⁵¹Cr) release assay in which MHC compatible target cells were infected with either

recombinant adenovirus or vaccinia viruses that express single relevant gene products. Figure 10 presents both an example of a ⁵¹Cr release assay in which % specific lysis is measured as a function of increasing the effector to target ratio (Fig. 10B), as well as a summary of the cumulative data (Fig. 10A). Splenocytes from C57BL/6 mice infused with recombinant adenovirus containing either human LDL receptor or human CFTR were evaluated for their ability to lyse targets infected with either recombinant adenovirus, to measure activity to viral proteins, or with vaccinia virus containing LDL receptor, to measure activity to LDL receptor protein. Cytolytic activity was demonstrated with lymphocytes from animals infected with the LDL receptor virus to target cells infected with the same virus. No cytolysis was detected to mock infected targets supporting the specificity of the assay. These same effector cells demonstrated significant cytolytic activity to targets infected with LDL receptor vaccinia virus that was not present when infected with a control vaccinia. These experiments provide strong evidence for the presence of activated CTL to human LDL receptor in C57BL/6 mice following gene therapy.

25 Example 9 - Enhancement of Adenovirus Mediated Gene Transfer upon Second Administration by IL-12 and IFN- γ in Mouse Lung.

The recombinant adenoviruses H5.010CMVlacZ and H5.010CBALP (alkaline phosphatase gene expressed from the CMV enhanced β -actin promoter in the sub360 backbone) were used in this example. Each similar virus expresses a different reporter gene whose expression can be discriminated from that of the first reporter gene.

Female C57Bl/6 mice (6-8 week old) were infected with suspensions of H5.010CBALP (1×10^9 pfu in 50 μ l of PBS) via the trachea at day 0 and similarly with H5.010CMVlacZ at day 28. One group of such mice was used
5 as a control. Another group of mice were acutely depleted of CD4 $^{+}$ cells by i.p. injection of antibody to CD4 $^{+}$ cells (GK1.5; ATCC No. TIB207, 1:10 dilution of ascites) at the time of the initial gene therapy (days - 3, 0, and +3). A third group of mice were injected with
10 IL-12 (1 μ g intratracheal or 2 μ g, i.p. injections) at the time of the first administration of virus (days 0 and +1). A fourth group of mice were injected with gamma interferon (1 μ g intratracheal or 2 μ g, i.p. injections) at the time of the first administration of virus (days 0
15 and +1).

When mice were subsequently euthanized and necropsied at days 3, 28, or 31, lung tissues were prepared for cryosections, while bronchial alveolar lavage (BAL) and mediastinal lymph nodes (MLN) were harvested for immunological assays.
20

A. Cryosections

The lung tissues were evaluated for alkaline phosphatase expression by histochemical staining following the procedures of Y. Yang et al, cited above.
25

Instillation of alkaline phosphatase virus (10^9 pfu) into the airway of all groups of the C57Bl/6 mice resulted in high level transgene expression in the majority of conducting airways that diminishes to undetectable levels by day 28. Loss of transgene
30 expression was shown to be due to CTL mediated elimination of the genetically modified hepatocytes [Y. Yang et al, cited above].

In the control mice, no recombinant gene expression was detected three days after the second
35 administration of virus, i.e., day 31.

Administration of virus to the CD4⁺ depleted animals was associated with high level recombinant transgene expression that was stable for a month. Expression of the second virus was detectable on 5 day 31.

Initial high level gene transfer diminished after about one month in the IL-12 treated mice; however, in contrast to the control, high level gene transfer to airway epithelial cells was achieved 10 when virus was readministered to IL-12 treated animals at day 28, as seen in the day 31 results.

The gamma-interferon treated animals were virtually indistinguishable from the animals treated with IL-12 in that efficient gene transfer was accomplished 15 upon a second administration of virus.

B. Immunological Assays - MLN

Lymphocytes from MLN of the control group and IL-12 treated group of C57Bl/6 mice harvested 28 days after administration of H5.010CBALP were restimulated in 20 vitro with UV-inactivated H5.010CMVlacZ at 10 particles/cell for 24 hours. Cell-free supernatants were assayed for the presence of IL-2 or IL-4 on HT-2 cells (an IL-2 or IL-4-dependent cell line) [Y. Yang et al, cited above]. Presence of IFN- γ in the same lymphocyte culture 25 supernatant was measured on L929 cells as described [Y. Yang et al, cited above]. Stimulation index (S.I.) was calculated by dividing 3 H-thymidine cpm incorporated into HT-2 cells cultured in supernatants of lymphocytes restimulated with virus by those incorporated into HT-2 30 cells cultured in supernatants of lymphocytes incubated in antigen-free medium.

The results are shown in Table 1 below.

Table 1

		<u>³H-Thymidine Incorporation (cpm±SD)</u>	<u>IFN-γ liter (IU/ml)^a</u>
	Medium	H5.010CMVlacZ S.I.	
5	C57Bl/6	175 ± 40	2084 ± 66
	anti-IL2 (1:5000)		11.91
		523 ± 81	80
10	anti-IL4 (1:5000)		2.98
		1545 ± 33	8.83
	C57Bl/6 +IL12	247 ± 34	5203 ± 28
15	anti-IL2 (1:5000)		21.07
		776 ± 50	160
	anti-IL4 (1:5000)		3.14
		4608 ± 52	18.66

Stimulation of lymphocytes from regional lymph nodes with both recombinant adenoviruses led to secretion of cytokines specific for the activation of both T_{H1} (i.e., IL-2 and IFN- γ) and T_{H2} (i.e., IL-4) subsets of T helper cells (Table 1).

Analysis of lymphocytes from the IL-12 treated animals stimulated *in vitro* with virus revealed an increased secretion of IL-2 and IFN- γ and a relative decreased production of IL-4 as compared to animals that did not receive IL-12 (i.e., ratio of IL-2/IL-4 was increased from 3 to 6 when IL-12 was used; Table 1).

30 C. Immunological Assays - BAL

BAL samples obtained from animals 28 days after primary exposure to recombinant virus were evaluated for neutralizing antibodies to adenovirus and anti-adenovirus antibody isotypes as follows. The same four groups of C57Bl/6 mice, i.e., control, CD4⁺

depleted, IL-12 treated and IFN- γ treated, were infected with H5.010CBALP. Neutralizing antibody was measured in serially diluted BAL samples (100 μ l) which were mixed with H5.010CMVlacZ (1×10^6 pfu in 20 μ l), incubated for 5 1 hour at 37°C, and applied to 80% confluent HeLa cells in 96 well plates (2×10^4 cells per well). After 60 minutes of incubation at 37°C, 100 μ l of DMEM containing 20% FBS was added to each well. Cells were fixed and stained for β -galactosidase expression the following day.

10 All cells were lacZ positive in the absence of anti-adenoviral antibodies.

Adenovirus-specific antibody isotype was determined in BAL by using enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates were coated with 15 100 μ l of PBS containing 5×10^9 particles of H5.010CMVlacZ for 18 hours at 4°C. The wells were washed 5 times with PBS. After blocking with 200 μ l of 2% BSA in PBS, the plates were rinsed once with PBS and incubated with 1:10 diluted BAL samples for 90 minutes at 20 4°C. Thereafter, the wells were extensively washed and refilled with 100 μ l of 1:1000 diluted alkaline phosphatase-conjugated anti-mouse IgG or IgA (Sigma). The plates were incubated, subsequently washed 5 times, and 100 μ l of the substrate solution (p-nitrophenyl 25 phosphate, PNPP) was added to each well. Substrate conversion was stopped by the addition of 50 μ l of 0.1M EDTA. Plates were read at 405 nm.

The results are shown graphically in Figs. 11A through 11C, which summarize neutralizing antibody titer, and the relative amounts (OD_{405}) of IgG and IgA present in BAL samples. The titer of neutralizing antibody for each sample was reported as the highest dilution with which less than 50% of cells stained blue.

As demonstrated in the first bar of Figs. 11A through 11C, the cytokines identified in Table 1 above were associated in the control mice with the appearance of antibodies to adenovirus proteins in BAL of 5 both the IgG and IgA isotypes that were capable of neutralizing the human Ad5 recombinant vector in an *in vitro* assay out to a 1:800 dilution.

As shown in the second bar of the graphs of Figs. 11A through 11C, transient CD4⁺ cell depletion 10 inhibited the formation of neutralizing antibody (Fig. 11A) and virus specific IgA antibody (Fig. 11C) by 80-fold, thereby allowing efficient gene transfer to occur following a second administration of virus. Fig. 11B shows a slight inhibition of IgG as well.

More importantly, as shown in the third 15 bar of the three graphs, IL-12 selectively blocked secretion of antigen specific IgA (Fig. 11C), without significantly impacting on formation of IgG (Fig. 11B). This was concurrent with a 32-fold reduction in 20 neutralizing antibody (Fig. 11A).

The gamma-interferon treated animals (fourth bar of Figs. 11A through 11B) were virtually indistinguishable from the animals treated with IL-12 in 25 that virus specific IgA (Fig. 11C) and neutralizing antibody (Fig. 11A) were decreased as compared to the control animals not treated with cytokine, but not to the extent obtained with those treated with IL-12.

These studies demonstrate that inhibition 30 of CD4⁺ function at the time of primary exposure to virus is sufficient to prevent the formation of blocking antibodies. The concordant reduction of neutralizing antibody with antiviral IgA suggests that immunoglobulin of the IgA subtype is primarily responsible for the blockade to gene transfer.

All references recited above are incorporated herein by reference. Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention, such as selections of different modifications of adenovirus vectors selected to carry the VLDLR gene, or selection or dosage of the vectors or immune modulators are believed to be within the scope of the claims appended hereto.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Trustees of University of Pennsylvania
Wilson, James M.
Kozarsky, Karen F.
Strauss, Jerome F.

(ii) TITLE OF INVENTION: Methods and Compositions for Gene Therapy for the Treatment of Defects in Lipoprotein Metabolism

(iii) NUMBER OF SEQUENCES: 8

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(A) ADDRESSEE: Howson and Howson
(B) STREET: Spring House Corporate Cntr., PO Box 457
(C) CITY: Spring House
(D) STATE: Pennsylvania
(E) COUNTRY: USA
(F) ZIP: 19477

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/393,734
(B) FILING DATE: 24-FEB-1995

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Bak, Mary E.
(B) REGISTRATION NUMBER: 31,215
(C) REFERENCE/DOCKET NUMBER: GNVN009CIP1.PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 215-540-9200
(B) TELEFAX: 215-540-5818

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3656 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 392..3010

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCTGCGGGC CGCGGGTGC GGTGCGCTGC ACCGGCTCTC TCCGTTCTGT GCTCTCTTCT	60
GCTCTCGGCT CCCCCACCCCCC TCTCCCTTCC CTCCCTCTCCC CTTGCCTCCC CTCCCTCTGCA	120
GCGCCTGCCAT TATTTTCTGC CGCGAGCTCG GCTTGCACTG CTGCTGCCAGC CCGGGGAGGT	180
GGCTGGGTGG GTGGGGAGGA GACTGTGCAA GTTGTAGGGG AGGGGGTGCC CTCTTCTTCC	240
CCGCTCCCTT CCCCCAGCCAA GTGGTTCCCC TCCTTCTCCC CCTTTCCCCT CCCAGCCCCC	300
ACCTTCTTCC TCTTTGGAA GGGCTGGTAA CTTGTCGTGC GGAGCGAACG CGGGCGGCCG	360
CGGCGGCCGC GGCACCATCC AGGCAGGGCAC C ATG GGC ACG TCC GCG CTC TGG Met Gly Thr Ser Ala Leu Trp	412
1 5	
GCC GTC TGG CTG CTG CTC GCG CTG TGC TGG GCG CCC CGG GAG AGC GGC Ala Val Trp Leu Leu Ala Leu Cys Trp Ala Pro Arg Glu Ser Gly	460
10 15 20	
GCC ACC GGA ACC GGG AGA AAA GCC AAA TGT GAA CCC TCC CAA TTC CAG Ala Thr Gly Thr Gly Arg Lys Ala Lys Cys Glu Pro Ser Gln Phe Gln	508
25 30 35	
TGC ACA AAT GGT CGC TGT ATT ACG CTG TTG TGG AAA TGT GAT GGG GAT Cys Thr Asn Gly Arg Cys Ile Thr Leu Leu Trp Lys Cys Asp Gly Asp	556
40 45 50 55	
GAA GAC TGT GTT GAC GGC AGT GAT GAA AAG AAC TGT GTA AAG AAG ACG Glu Asp Cys Val Asp Gly Ser Asp Glu Lys Asn Cys Val Lys Lys Thr	604
60 65 70	
TGT GCT GAA TCT GAC TTC GTG TGC AAC AAT GGC CAG TGT GTT CCC AGC Cys Ala Glu Ser Asp Phe Val Cys Asn Asn Gly Gln Cys Val Pro Ser	652
75 80 85	
CGA TGG AAG TGT GAT GGA GAT CCT GAC TGC GAA GAT GGT TCA GAT GAA Arg Trp Lys Cys Asp Gly Asp Pro Asp Cys Glu Asp Gly Ser Asp Glu	700
90 95 100	
AGC CCA GAA CAG TGC CAT ATG AGA ACA TGC CGC ATA CAT GAA ATC AGC Ser Pro Glu Gln Cys His Met Arg Thr Cys Arg Ile His Glu Ile Ser	748
105 110 115	
TGT GGC GCC CAT TCT ACT CAG TGT ATC CCA GTG TCC TGG AGA TGT GAT Cys Gly Ala His Ser Thr Gln Cys Ile Pro Val Ser Trp Arg Cys Asp	796
120 125 130 135	
GGT GAA AAT GAT TGT GAC AGT GGA GAA GAT GAA GAA AAC TGT GGC AAT Gly Glu Asn Asp Cys Asp Ser Gly Glu Asp Glu Glu Asn Cys Gly Asn	844
140 145 150	
ATA ACA TGT AGT CCC GAC AG TTC ACC TGC TCC AGT GGC CGC TGC ATC Ile Thr Cys Ser Pro Asp Glu Phe Thr Cys Ser Ser Gly Arg Cys Ile	892
155 160 165	

TCC AGG AAC TTT GTA TGC AAT GGC CAG GAT GAC TGC AGC GAT GGC AGT Ser Arg Asn Phe Val Cys Asn Gly Gln Asp Asp Cys Ser Asp Gly Ser 170	175	180	940
GAT GAG CTG GAC TGT GCC CCG CCA ACC TGT GGC GCC CAT GAG TTC CAG Asp Glu Leu Asp Cys Ala Pro Pro Thr Cys Gly Ala His Glu Phe Gln 185	190	195	988
TGC AGC ACC TCC TCC TGC ATC CCC ATC AGC TGG GTA TGC GAC GAT GAT Cys Ser Thr Ser Ser Cys Ile Pro Ile Ser Trp Val Cys Asp Asp Asp 200	205	210	215
GCA GAC TGC TCC GAC CAA TCT GAT GAG TCC CTG GAG CAG TGT GGC CGT Ala Asp Cys Ser Asp Gln Ser Asp Glu Ser Leu Glu Gln Cys Gly Arg 220	225	230	1084
CAG CCA GTC ATA CAC ACC AAG TGT CCA GCC AGC GAA ATC CAG TGC GGC Gln Pro Val Ile His Thr Lys Cys Pro Ala Ser Glu Ile Gln Cys Gly 235	240	245	1132
TCT GGC GAG TGC ATC CAT AAG AAG TGG CGA TGT GAT GGG GAC CCT GAC Ser Gly Glu Cys Ile His Lys Lys Trp Arg Cys Asp Gly Asp Pro Asp 250	255	260	1180
TCC AAG GAT GGC AGT GAT GAG GTC AAC TGT CCC TCT CGA ACT TGC CGA Cys Lys Asp Gly Ser Asp Glu Val Asn Cys Pro Ser Arg Thr Cys Arg 265	270	275	1228
CCT GAC CAA TTT GAA TGT GAG GAT GGC AGC TGC ATC CAT GGC AGC AGG Pro Asp Gln Phe Glu Cys Glu Asp Gly Ser Cys Ile His Gly Ser Arg 280	285	290	1276
CAG TGT AAT GGT ATC CGA GAC TGT GTC GAT GGT TCC GAT GAA GTC AAC Gln Cys Asn Gly Ile Arg Asp Cys Val Asp Gly Ser Asp Glu Val Asn 300	305	310	1324
TGC AAA AAT GTC AAT CAG TGC TTG GGC CCT GGA AAA TTC AAG TGC AGA Cys Lys Asn Val Asn Gln Cys Leu Gly Pro Gly Lys Phe Lys Cys Arg 315	320	325	1372
AGT GGA GAA TGC ATA GAT ATC AGC AAA GTA TGT AAC CAG GAG CAG GAC Ser Gly Glu Cys Ile Asp Ile Ser Lys Val Cys Asn Gln Glu Gln Asp 330	335	340	1420
TGC AGG GAC TGG AGT GAT GAG CCC CTG AAA GAG TGT CAT ATA AAC GAA Cys Arg Asp Trp Ser Asp Glu Pro Leu Lys Glu Cys His Ile Asn Glu 345	350	355	1468
TGC TTG GTC AAT AAT GGT GGA TGT TCT CAT ATC TGC AAA GAC CTA GTT Cys Leu Val Asn Asn Gly Gly Cys Ser His Ile Cys Lys Asp Leu Val 360	365	370	1516
ATA GGC TAC GAG TGT GAC TGT GCA GCT GGG TTT GAA CTG ATA GAT AGG Ile Gly Tyr Glu Cys Asp Cys Ala Ala Gly Phe Glu Leu Ile Asp Arg 380	385	390	1564
AAA ACC TGT GGA GAT ATT GAT AA TGC CAA AAT CCA GGA ATC TGC AGT Lys Thr Cys Gly Asp Ile Asp Glu Cys Gln Asn Pro Gly Ile Cys Ser 395	400	405	1612

CAA ATT TGT ATC AAC TTA AAA GGC GGT TAC AAG TGT GAA TGT AGT CGT Gln Ile Cys Ile Asn Leu Lys Gly Gly Tyr Lys Cys Glu Cys Ser Arg 410 415 420	1660
GCC TAT CAA ATG GAT CTT GCT ACT GGC GTG TGC AAG GCA GTA GGC AAA Ala Tyr Gln Met Asp Leu Ala Thr Gly Val Cys Lys Ala Val Gly Lys 425 430 435	1708
GAG CCA AGT CTG ATC TTC ACT AAT CGA AGA GAC ATC AGG AAG ATT GGC Glu Pro Ser Leu Ile Phe Thr Asn Arg Arg Asp Ile Arg Lys Ile Gly 440 445 450 455	1756
TTA GAG AGG AAA GAA TAT ATC CAA CTA GTT GAA CAG CTA AGA AAC ACT Leu Glu Arg Lys Glu Tyr Ile Gln Leu Val Glu Gln Leu Arg Asn Thr 460 465 470	1804
GTG GCT CTC GAT GCT GAC ATT GCT GCC CAG AAA CTA TTC TGG GCC GAT Val Ala Leu Asp Ala Asp Ile Ala Gln Lys Leu Phe Trp Ala Asp 475 480 485	1852
CTA AGC CAA AAG GCT ATC TTC AGT GCC TCA ATT GAT GAC AAG GTT GGT Leu Ser Gln Lys Ala Ile Phe Ser Ala Ser Ile Asp Asp Lys Val Gly 490 495 500	1900
AGA CAT GTT AAA ATG ATC GAC AAT GTC TAT AAT CCT GCA GCC ATT GCT Arg His Val Lys Met Ile Asp Asn Val Tyr Asn Pro Ala Ala Ile Ala 505 510 515	1948
GTT GAT TGG GTG TAC AAG ACC ATC TAC TGG ACT GAT GCG GCT TCT AAG Val Asp Trp Val Tyr Lys Thr Ile Tyr Trp Thr Asp Ala Ala Ser Lys 520 525 530 535	1996
ACT ATT TCA GTA GCT ACC CTA GAT GGA ACC AAG AGG AAG TTC CTG TTT Thr Ile Ser Val Ala Thr Leu Asp Gly Thr Lys Arg Lys Phe Leu Phe 540 545 550	2044
AAC TCT GAC TTG CGA GAG CCT GCC TCC ATA GCT GTG GAC CCA CTG TCT Asn Ser Asp Leu Arg Glu Pro Ala Ser Ile Ala Val Asp Pro Leu Ser 555 560 565	2092
GGC TTT GTT TAC TGG TCA GAC TGG GGT GAA CCA GCT AAA ATA GAA AAA Gly Phe Val Tyr Trp Ser Asp Trp Gly Glu Pro Ala Lys Ile Glu Lys 570 575 580	2140
GCA GGA ATG AAT GGA TTC GAT AGA CGT CCA CTG GTG ACA GCG GAT ATC Ala Gly Met Asn Gly Phe Asp Arg Arg Pro Leu Val Thr Ala Asp Ile 585 590 595	2188
CAG TGG CCT AAC GGA ATT ACA CTT GAC CTT ATA AAA AGT CGC CTC TAT Gln Trp Pro Asn Gly Ile Thr Leu Asp Leu Ile Lys Ser Arg Leu Tyr 600 605 610 615	2236
TGG CTT GAT TCT AAG TTG CAC ATG TTA TCC AGC GTG GAC TTG AAT GGC Trp Leu Asp Ser Lys Leu His Met Leu Ser Ser Val Asp Leu Asn Gly 620 625 630	2284
CAA GAT CGT AGG ATA GTA CTA AAG TCT CTG GAG TTC CTA GCT CAT CCT Gln Asp Arg Arg Ile Val Leu Lys Ser Leu Ile Phe Leu Ala His Pro 635 640 645	2332
CTT GCA CTA ACA ATA TTT GAG GAT CGT GTC TAC TGG ATA GAT GGG GAA Leu Ala Leu Thr Ile Phe Glu Asp Arg Val Tyr Trp Ile Asp Gly Glu 650 655 660	2380

65

AAT GAA GCA TC TAT GGT GCC AAT AAA TTC ACT GGA TCA GAG CAT GCC Asn Glu Ala Val Tyr Gly Ala Asn Lys Phe Thr Gly Ser Glu His Ala 665 670 675	2428
ACT CTA GTC AAC AAC CTG AAT GAT GCC CAA GAC ATC ATT GTC TAT CAT Thr Leu Val Asn Asn Leu Asn Asp Ala Gln Asp Ile Ile Val Tyr His 680 685 690 695	2476
GAA CTT GTA CAG CCA TCA GGT AAA AAT TGG TGT GAA GAA GAC ATG GAG Glu Leu Val Gln Pro Ser Gly Lys Asn Trp Cys Glu Glu Asp Met Glu 700 705 710	2524
AAT GGA GGA TGT GAA TAC CTA TGC CTG CCA GCA CCA CAG ATT AAT GAT Asn Gly Gly Cys Glu Tyr Leu Cys Leu Pro Ala Pro Gln Ile Asn Asp 715 720 725	2572
CAC TCT CCA AAA TAT ACC TGT TCC TGT CCC AGT GGG TAC AAT GTA GAG His Ser Pro Lys Tyr Thr Cys Ser Cys Pro Ser Gly Tyr Asn Val Glu 730 735 740	2620
GAA AAT GGC CGA GAC TGT CAA AGT ACT GCA ACT ACT GTG ACT TAC AGT Glu Asn Gly Arg Asp Cys Gln Ser Thr Ala Thr Thr Val Thr Tyr Ser 745 750 755	2668
GAG ACA AAA GAT ACG AAC ACA ACA GAA ATT TCA GCA ACT AGT GGA CTA Glu Thr Lys Asp Thr Asn Thr Glu Ile Ser Ala Thr Ser Gly Leu 760 765 770 775	2716
GTT CCT GGA GGG ATC AAT GTG ACC ACA GCA GTA TCA GAG GTC AGT GTT Val Pro Gly Gly Ile Asn Val Thr Ala Val Ser Glu Val Ser Val 780 785 790	2764
CCC CCA AAA GGG ACT TCT GCC GCA TGG GCC ATT CTT CCT CTC TTG CTC Pro Pro Lys Gly Thr Ser Ala Ala Trp Ala Ile Leu Pro Leu Leu 795 800 805	2812
TTA GTG ATG GCA GCA GTA GGT GGC TAC TTG ATG TGG CGG AAT TGG CAA Leu Val Met Ala Ala Val Gly Gly Tyr Leu Met Trp Arg Asn Trp Gln 810 815 820	2860
CAC AAG AAC ATG AAA AGC ATG AAC TTT GAC AAT CCT GTG TAC TTG AAA His Lys Asn Met Lys Ser Met Asn Phe Asp Asn Pro Val Tyr Leu Lys 825 830 835	2908
ACC ACT GAA GAG GAC CTC TCC ATA GAC ATT GGT AGA CAC AGT GCT TCT Thr Thr Glu Glu Asp Leu Ser Ile Asp Ile Gly Arg His Ser Ala Ser 840 845 850 855	2956
GTT CGA CAC ACG TAC CCA GCA ATA TCA GTT GTA AGC ACA GAT GAT GAT Val Gly His Thr Tyr Pro Ala Ile Ser Val Val Ser Thr Asp Asp Asp 860 865 870	3004
CTA GCT TGACTTCTGT GACAAATGTT GACCTTGAG GTCTAACAA ATAATAACCC Leu Ala	3060
CGTCGGAATG GTAACCGAGC CAGCAGCTGA AGTCTCTTT TCTTCCTCTC GGCTGGAAAGA	3120
ACATCAAGAT ACCTTTGGGT GGATCAAGCT TGCTGTACTT GACCGTTTTT ATATTACTTT	3180
TGTAAATATT CTTGTCCACA TTCTACTTCA GCTTTGGATG TGGTTACCGA GTATCTGAA	3240
CCCTTGAATT TCTAGACAGT ATTGCCACCT CTGGCCAAAT ATGCACTTTC CCTAGAAAGC	3300

CATATTCCAG CAGTGAAACT TGTGCTATAAG TGTATAACCAAC CTGTACATAC ATTGTATAGG	3360
CCATCTGTAA ATATCCCAGA GAACAATCAC TATTCTTAAG CACTTTGAAA ATATTTCTAT	3420
GTAAATTATT GTAAACCTTT TCAATGGTTG GGACAATGGC AATAGGACAA AACGGGTTAC	3480
TAAGATGAAA TTGCCAAAAA AATTTATAAA CTAATTTTGG TACGTATGAA TGATATCTTT	3540
GACCTCAATG GAGGTTTGCA AAGACTGAGT GTTCAAACTA CTGTACATTT TTTTCAGT	3600
GCTAAAAAAAT TAAACCAAGC AGCTTAAAAA AAAAAAAA AAAAAAAA AAAAAA	3656

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 873 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Gly Thr Ser Ala Leu Trp Ala Val Trp Leu Leu Leu Ala Leu Cys	
1 5 10 15	
Trp Ala Pro Arg Glu Ser Gly Ala Thr Gly Thr Gly Arg Lys Ala Lys	
20 25 30	
Cys Glu Pro Ser Gln Phe Gln Cys Thr Asn Gly Arg Cys Ile Thr Leu	
35 40 45	
Leu Trp Lys Cys Asp Gly Asp Glu Asp Cys Val Asp Gly Ser Asp Glu	
50 55 60	
Lys Asn Cys Val Lys Lys Thr Cys Ala Glu Ser Asp Phe Val Cys Asn	
65 70 75 80	
Asn Gly Gln Cys Val Pro Ser Arg Trp Lys Cys Asp Gly Asp Pro Asp	
85 90 95	
Cys Glu Asp Gly Ser Asp Glu Ser Pro Glu Gln Cys His Met Arg Thr	
100 105 110	
Cys Arg Ile His Glu Ile Ser Cys Gly Ala His Ser Thr Gln Cys Ile	
115 120 125	
Pro Val Ser Trp Arg Cys Asp Gly Glu Asn Asp Cys Asp Ser Gly Glu	
130 135 140	
Asp Glu Glu Asn Cys Gly Asn Ile Thr Cys Ser Pro Asp Glu Phe Thr	
145 150 155 160	
Cys Ser Ser Gly Arg Cys Ile Ser Arg Asn Phe Val Cys Asn Gly Gln	
165 170 175	
Asp Asp Cys Ser Asp Gly Ser Asp Glu Leu Asp Cys Ala Pro Pro Thr	
180 185 190	

Cys Gly Ala His Glu Phe Gln Cys Ser Thr Ser Ser Cys Ile Pro Ile
195 200 205

Ser Trp Val Cys Asp Asp Asp Ala Asp Cys Ser Asp Gln Ser Asp Glu
210 215 220

Ser Leu Glu Gln Cys Gly Arg Gln Pro Val Ile His Thr Lys Cys Pro
225 230 235 240

Ala Ser Glu Ile Gln Cys Gly Ser Gly Glu Cys Ile His Lys Lys Trp
245 250 255

Arg Cys Asp Gly Asp Pro Asp Cys Lys Asp Gly Ser Asp Glu Val Asn
260 265 270

Cys Pro Ser Arg Thr Cys Arg Pro Asp Gln Phe Glu Cys Glu Asp Gly
275 280 285

Ser Cys Ile His Gly Ser Arg Gln Cys Asn Gly Ile Arg Asp Cys Val
290 295 300

Asp Gly Ser Asp Glu Val Asn Cys Lys Asn Val Asn Gln Cys Leu Gly
305 310 315 320

Pro Gly Lys Phe Lys Cys Arg Ser Gly Glu Cys Ile Asp Ile Ser Lys
325 330 335

Val Cys Asn Gln Glu Gln Asp Cys Arg Asp Trp Ser Asp Glu Pro Leu
340 345 350

Lys Glu Cys His Ile Asn Glu Cys Leu Val Asn Asn Gly Gly Cys Ser
355 360 365

His Ile Cys Lys Asp Leu Val Ile Gly Tyr Glu Cys Asp Cys Ala Ala
370 375 380

Gly Phe Glu Leu Ile Asp Arg Lys Thr Cys Gly Asp Ile Asp Glu Cys
385 390 395 400

Gln Asn Pro Gly Ile Cys Ser Gln Ile Cys Ile Asn Leu Lys Gly Gly
405 410 415

Tyr Lys Cys Glu Cys Ser Arg Ala Tyr Gln Met Asp Leu Ala Thr Gly
420 425 430

Val Cys Lys Ala Val Gly Lys Glu Pro Ser Leu Ile Phe Thr Asn Arg
435 440 445

Arg Asp Ile Arg Lys Ile Gly Leu Glu Arg Lys Glu Tyr Ile Gln Leu
450 455 460

Val Glu Gln Leu Arg Asn Thr Val Ala Leu Asp Ala Asp Ile Ala Ala
465 470 475 480

Gln Lys Leu Phe Trp Ala Asp Leu Ser Gln Lys Ala Ile Phe Ser Ala
485 490 495

Ser Ile Asp Asp Lys Val Gly Arg His Val Lys Met Ile Asp Asn Val
500 505 510

Tyr Asn Pro Ala Ala Ile Ala Val Asp Trp Val Tyr Lys Thr Ile Tyr
515 520 525

68

Trp Thr Asp Ala Ala Ser Lys Thr Ile Ser Val Ala Thr Leu Asp Gly
 530 535 540
 Thr Lys Arg Lys Phe Leu Phe Asn Ser Asp Leu Arg Glu Pro Ala Ser
 545 550 555 560
 Ile Ala Val Asp Pro Leu Ser Gly Phe Val Tyr Trp Ser Asp Trp Gly
 565 570 575
 Glu Pro Ala Lys Ile Glu Lys Ala Gly Met Asn Gly Phe Asp Arg Arg
 580 585 590
 Pro Leu Val Thr Ala Asp Ile Gln Trp Pro Asn Gly Ile Thr Leu Asp
 595 600 605
 Leu Ile Lys Ser Arg Leu Tyr Trp Leu Asp Ser Lys Leu His Met Leu
 610 615 620
 Ser Ser Val Asp Leu Asn Gly Gln Asp Arg Arg Ile Val Leu Lys Ser
 625 630 635 640
 Leu Glu Phe Leu Ala His Pro Leu Ala Leu Thr Ile Phe Glu Asp Arg
 645 650 655
 Val Tyr Trp Ile Asp Gly Glu Asn Glu Ala Val Tyr Gly Ala Asn Lys
 660 665 670
 Phe Thr Gly Ser Glu His Ala Thr Leu Val Asn Leu Asn Asp Ala
 675 680 685
 Gln Asp Ile Ile Val Tyr His Glu Leu Val Gln Pro Ser Gly Lys Asn
 690 695 700
 Trp Cys Glu Glu Asp Met Glu Asn Gly Gly Cys Glu Tyr Leu Cys Leu
 705 710 715 720
 Pro Ala Pro Gln Ile Asn Asp His Ser Pro Lys Tyr Thr Cys Ser Cys
 725 730 735
 Pro Ser Gly Tyr Asn Val Glu Glu Asn Gly Arg Asp Cys Gln Ser Thr
 740 745 750
 Ala Thr Thr Val Thr Tyr Ser Glu Thr Lys Asp Thr Asn Thr Thr Glu
 755 760 765
 Ile Ser Ala Thr Ser Gly Leu Val Pro Gly Gly Ile Asn Val Thr Thr
 770 775 780
 Ala Val Ser Glu Val Ser Val Pro Pro Lys Gly Thr Ser Ala Ala Trp
 785 790 795 800
 Ala Ile Leu Pro Leu Leu Leu Val Met Ala Ala Val Gly Gly Tyr
 805 810 815
 Leu Met Trp Arg Asn Trp Gln His Lys Asn Met Lys Ser Met Asn Phe
 820 825 830
 Asp Asn Pro Val Tyr Leu Lys Thr Thr Glu Glu Asp Leu Ser Ile Asp
 835 840 845

Ile Gly Arg His Ser Ala Ser Val Gly His Thr Tyr Pr Ala Ile Ser
 850 855 860

Val Val Ser Thr Asp Asp Asp Leu Ala
 865 870

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 9592 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAATTTCGCTA	GCATCATCAA	TAATATAACCT	TATTTGGAT	TGAAGCCAAT	ATGATAATGA	60
GGGGGTGGAG	TTTGTGACGT	GGCGCGGGGC	GTGGGAACGG	GGCGGGTGAC	GTAGTAGTGT	120
GGCGGAAGTG	TGATGTTGCA	AGTGTGGCGG	AACACATGTA	AGCGACGGAT	GTGGCAAAAG	180
TGACGTTTTT	GGTGTGCC	GGTGTACACA	GGAAGTGACA	ATTTCGCGC	GGTTTTAGGC	240
GGATGTTGTA	GTAAATTG	GGCTAACCGA	CTAAGATTTG	GCCATTTCG	CGGGAAAACT	300
GAATAAGAGG	AACTGAAATC	TGAATAATT	TGTGTTACTC	ATAGCCGTA	ATATTGTCT	360
AGGGAGATCA	GCCTGCAGGT	CGTTACATAA	CTTACGGTAA	ATGGCCCGCC	TGGCTGACCG	420
CCCAACGACC	CCCGCCCCATT	GACGTCAATA	ATGACGTATG	TTCCCATAGT	AACGCCAATA	480
GGGACTTCC	ATTGACGTCA	ATGGGTGGAG	TATTTACGGT	AAACTGCCA	CTTGGCAGTA	540
CATCAAGTGT	ATCATATGCC	AACTACGCC	CCTATTGACG	TCAATGACGG	TAATGGCCC	600
GCCTGGCATT	ATGCCAGTA	CATGACCTTA	TGGACTTTC	CTACTTGGCA	GTACATCTAC	660
GTATTAGTCA	TCGCTATTAC	CATGGTGATG	CGGTTTGGC	AGTACATCAA	TGGCGTGGA	720
TAGCGGTTG	ACTCACGGGG	ATTTCCAAGT	CTCCACCCCA	TTGACGTCAA	TGGGAGTTG	780
TTTGGCACC	AAAATCAACG	GGACTTCCA	AAATGTCGTA	ACAACCTCCG	CCCATTGACG	840
CAAATGGGCC	GTAGGCCTGT	ACGGTGGAG	GTCTATATAA	GCAGAGCTCT	CTGGCTAACT	900
AGAGAACCCA	CTGCTTAACT	GGCTTATCGA	AATTAATACG	ACTCACTATA	GGGAGACCCA	960
AGCTTCTCTG	CGGGCCGGG	GTGGGGTGTG	TCGCTACCGG	CTCTCTCCGT	TCTGTGCTCT	1020
CTTCTGCTCT	CGGCTCCCCA	CCCCCTCTCC	CTTCCCTCCT	CTCCCCTTGC	CTCCCCCTCCT	1080
CTGCAGGCC	TGCATTATT	TCTGCCCGCA	GCTCGGCTTG	CACTGCTGCT	GCAGCCGGG	1140
GAGGTGGCTG	GGTGGGTGGG	GAGGAGACTG	TGCAAGTTGT	AGGGGAGGGG	GTGCCCTCTT	1200
CTTCCCCGCT	CCCTTCCCCA	GCCAAGTGGT	TCCCCCTCCTT	CTCCCCCTTT	CCCCCTCCCAG	1260

CCCCCACCTT	CTTCCCTTTT	CGCAAGGGCT	CGTAACCTGT	CGTCGGGAGC	GAACGGCGGC	1320
GGCGGCGGCG	CGGGCGGCAC	CATCCAGGGG	GGCACCATGG	GCACGTCCGC	GCTCTGGGCC	1380
GTCTGGCTGC	TGCTCGCGCT	GTGCTGGGGG	CCCCGGGAGA	CGGGCGCCAC	CGGAACCGGG	1440
AGAAAAGCCA	AATGTGAACC	CTCCCATTTC	CAGTGCACAA	ATGGTCGCTG	TATTACGCTG	1500
TTGTGAAAT	GTGATGGGG	TGAAGACTGT	GTTGACGGCA	GTGATGAAA	GAACGTGTA	1560
AAGAAGACGT	GTGCTGAATC	TGACTTCGTG	TGCAACAAATG	GCCAGTGTGT	TCCCAGCCGA	1620
TGGAAGTGTG	ATGGAGATCC	TGACTGCGAA	GATGGTTCA	ATGAAAGCCC	AGAACAGTGC	1680
CATATGAGAA	CATGCCGCAT	ACATGAAATC	AGCTGTGGCG	CCCATTCTAC	TCAGTGTATC	1740
CCAGTGTCT	GGAGATGTGA	TGGTGAAAT	GATTGTGACA	GTGGAGAAGA	TGAAGAAAAC	1800
TGTGGCAATA	TAACATGTAG	TCCCQACGAG	TTCACCTGCT	CCAGTGGCCG	CTGCATCTCC	1860
AGGAACCTTG	TATGCAATGG	CCAGGATGAC	TGCAGCGATG	GCAGTGTGA	GCTGCACTGT	1920
CCCCGCCAA	CCTGTGGCGC	CCATGAGTTC	CAGTGCAGCA	CCTCCTCCTG	CATCCCCATC	1980
AGCTGGGTAT	GGGACGATGA	TCCAGACTGC	TCCGACCAAT	CTGATGAGTC	CCTGGAGCAG	2040
TGTGGCGTC	AGCCAGTCAT	ACACACCAAG	TGTCCAGCCA	GCGAAATCCA	GTGGGGCTCT	2100
GGCGAGTGCA	TCCATAAGAA	GTGGCGATGT	GATGGGGACC	CTGACTGCAA	GGATGGCAGT	2160
GATGAGGTCA	ACTGTCCCTC	TGAACTTGC	CGACCTGACC	AATTGAAATG	TGAGGATGGC	2220
AGCTGCATCC	ATGGCAGCAG	GCAGTGTAA	GGTATCCGAG	ACTGTGTGCA	TGGTTCCGAT	2280
GAAGTCAACT	GCAAAAATGT	CAATCAGTGC	TTGGGCCCTG	AAAAATTCAA	GTGCAGAAGT	2340
GGAGAATGCA	TAGATATCAG	CAAAGTATGT	AACCAGGAGC	AGGACTGCAG	GGACTGGAGT	2400
GATGAGCCCC	TGAAAGAGTG	TCATATAAAC	GAATGCTTGG	TAATAATGG	TGGATGTTCT	2460
CATATCTGCA	AAGACCTAGT	TATAGGCTAC	GAGTGTGACT	GTGCAGCTGG	GTGAAACTG	2520
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ATTTGTATCA	ACTTAAAAGG	CGGTTACAAG	TGTGAATGTA	GTGCTGCCAA	TCAAATGGAT	2640
CTTGCTACTG	GGGTGTGCAA	GGCAGTAGGC	AAAGAGCCAA	GTCTGATCTT	CACTAATCGA	2700
AGAGACATCA	GGAAAGATTGG	CTTAGAGAGG	AAAGAATATA	TCCAACCTAGT	TGAACAGCTA	2760
AGAAAACACTG	TGGCTCTCGA	TGCTGACATT	GCTGCCAGA	AACTATTCTG	GGCCGATCTA	2820
AGCCAAAAGG	CTATCTTCAG	TGCCTCAATT	GATGACAAGG	TTGGTAGACAA	TGTTAAAATG	2880
ATCGACAATG	TCTATAATCC	TGCAGCCATT	GCTGTTGATT	GGGTGTACAA	GACCACATCTAC	2940
TGGACTGATG	CGGCTCTAA	GAATTTCA	GTAGCTACCC	TAGATGGAAC	CAAGAGGAAG	3000
TTCCCTGTTA	ACTCTGACTT	GGGAGAGCCT	GCCTCCATAG	CTGTGGACCC	ACTGTCTGGC	3060
TTTGTAACT	GGTCAGACTG	GGGTGAACCA	GCTAAAATAG	AAAAAGCAGG	AATGAATGGA	3120

TTCGATAGAC GTCCACTGGT GACAGCGGAT ATCCAGTGGC CTAACCGGAAT TACACTTGAC	3180
CTTATAAAAAA GTCGCCTCTA TTGGCTTGAT TCTAAGTTGC ACATGTTATC CAGCGTGGAC	3240
TTGAATGGCC AAGATCGTAG GATAGTACTA AAGTCTCTGG AGTTCCTAGC TCATCCTCTT	3300
GCACAAACAA TATTTGAGGA TCGTGTCTAC TGGATAGATG GGGAAAATGA AGCAGTCTAT	3360
GGTGCCAAATA AATTCACTGG ATCAGAGCAT GCCACTCTAG TCAACAAACCT GAATGATGCC	3420
CAAGACATCA TTGTCTATCA TGAACCTGTA CAGCCATCAG GTAAAAATTG GTGTGAAGAA	3480
GACATGGAGA ATGGAGGATG TGAATACCTA TGCCTGCCAG CACCACAGAT TAATGATCAC	3540
TCTCCAAAAT ATACCTGTTCTG CTGTCCCAGT GGGTACAATG TAGAGGAAAAA TGGCCGAGAC	3600
TGTCAAAGTA CTGCAACTAC TGTGACTTAG AGACAAAAGA TACGAACACA ACAGAAAATT	3660
CAGCAACTAG TGGAAGTAGTT CCTGGAGGGA TCAATGTGAC CACAGCAGTA TCAGAGGTCA	3720
GTGTTCCCCC AAAAGGGACT TCTGCCGCAT GGGCCATTCT TCCTCTCTTG CTCTTAGTGA	3780
TGGCAGCAGT AGGTGGCTAC TTGATGTGGC CGAATTGGCA ACACAAAGAAC ATGAAAAGCA	3840
TGAACCTTGA CAATCCTGTG TACTTGAAAA CCACTGAAGA GGACCTCTCC ATAGACATTG	3900
GTAGACACAG TGCTTCTGTT GGACACACGT ACCCAGCAAT ATCAGTTGTA AGCACAGATG	3960
ATGATCTAGC TTGACTTCTG TGACAAATGT TGACCTTTGA GGTCTAAACA AATAATACCC	4020
CCGTCGGAAT GGTAAACCGAG CCAGCAGCTG AAGTCTCTTT TTCTTCTCT CGGCTGGAAAG	4080
AACATCAAGA TACCTTTGCG TGGATCAAGC TTGGTACCGA GCTCGGATCC ACTAGTAACG	4140
GCGGCCAGTG TGCTGGAATT CTGCAGATAT CCATCACACT GGCGGCCGCG GGGATCCAGA	4200
CATGATAAGA TACATTGATG AGTTGGACA AACCAAACT AGAATGCAGT GAAAAAAATG	4260
CTTTATTGT GAAATTGTG ATGCTATTGC TTTATTGTA ACCATTATAA GCTGCAATAA	4320
ACAAGTTAAC AACAAACAATT GCATTCAATT TATGTTTCAG GTTCAGGGGG AGGTGTGGGA	4380
GGTTTTTCG GATCCTCTAG AGTCGACCTG CAGGCTGATC TGGAAAGGTGC TGAGGTACGA	4440
TGAGACCCGC ACCAGGTGCA GACCCCTGCGA CTGTGGCGGT AAACATATTA CGAACCGAGCC	4500
TGTGATGCTG GATGTGACCG AGGAGCTGAG GCCCCGATCAC TTGGTGTCTGG CCTGCACCCG	4560
CGCTGAGTTT GGCTCTAGCG ATGAAGATAC AGATTGAGGT ACTGAAATGT GTGGGCGTGG	4620
CTTAAGGGTG GGAAAGAATA TATAAGGTGG GGGTCTTATG TAGTTTGTA TCTGTTTGC	4680
AGCACCCGCC GCCGCCATGA GCACCAACTC GTTGTATGGA AGCATTTGTA GCTCATATT	4740
GACAACCGCGC ATGCCCCCAT GGGCCGGGGT GCGTCAGAAT GTGATGGCT CCAGCATTGA	4800
TGGTCGCCCC GTCCTGCCCG CAAACTCTAC TACCTTGACC TACGAGACCG TGTCTGGAAC	4860
GCCGTTGGAG ACTGCAGCCT CGGCCGCCGC TTCAGCCGCT GCAGCCACCG CCCGCCGGAT	4920
TGTGACTGAC TTTGCTTCC TGAGCCCGCT TGCAAGCAGT GCAGCTTCCC GTTCATCCGC	4980

CCCGATGAC AAGTTGACGG CTCTTTCGGC ACAATTGGAT TCTTTGACCC CGGAACCTAA	5040
TGTCTTTCT CAGCAGCTGT TGGATCTGGC CCAGCAGGTT TCTGCCCTGA AGGCTTCCTC	5100
CCCTCCCCAAAT GCGGTTAAA ACATAAATAA AAAACCCAGAC TCTGTTTGGA TTTGGATCAA	5160
GCAAGTGTCT TGCTGTCTTT ATTTAGGGGT TTTGCCGCC CGGTAGGCCCG GGGACCAGCG	5220
GTCTCGGTG TTGAGGGTCC TGTGTATTT TTCCAGGACG TGGTAAGGT GACTCTGGAT	5280
GTTCAGATAAC ATGGCATAA GCCCGTCTCT GGGGTGGAGG TAGCACCAC GCAAGAGCTTC	5340
ATGCTGCCGGG GTGGTGTGT AGATGATCCA GTCGTACGAG GAGCGCTGGG CGTGGTGCCT	5400
AAAAATGTCT TTCAGTAGCA AGCTGATTGC CAGGGGCAGG CCCTGGTGT AAGTGTAAAC	5460
AAAGCGGTTA AGCTGGGATG GGTGCATAAC TGGGGATATG AGATGCATCT TGGACTGTAT	5520
TTTTAGGTTG GCTATGTTCC CAGCCATATC CCTCCGGGGA TTCATGTTGT GCAGAACAC	5580
CACCAACAGTG TATCCGGTGC ACTTGGAAA TTTGTCAATGT AGCTTAAAG GAAATGCGTG	5640
GAAGAACTTG GAGACGCCCT TGTGACCTCC AAGATTTTCC ATGCATTCTGT CCATAATGAT	5700
GGCAATGGGC CCACGGGCGG CGGCCTGGC GAAGATATTT CTGGGATCAC TAACGTCATA	5760
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AGACTGCGGT ATAATGGTTC CATCCGGCCC AGGGGCGTAG TTACCCCTCAC AGATTTGCAT	5880
TTCCCCACGCT TTGAGTTCAAG ATGGGGGAT CATGTCTACC TGCGGGGCGA TGAAGAAAAC	5940
GGTTTCCGGG GTAGGGGAGA TCAGCTGGGA AGAAAGCAGG TTCCCTGAGCA CCTGCGACTT	6000
ACCGCAGCCG GTGGGGCGGT AAATCACACC TATTACCGGG TGCAACTGGT AGTTAAGAGA	6060
GCTGCAGCTG CCGTCATCCC TGAGCAGGGG GGCCACTTCG TTAAGCATGT CCCTGACTCG	6120
CATGTTTCC CTGACCAAAT CCGCCAGAAG CGCCTCGCCG CCCAGCGATA GCAGTTCTTG	6180
CAAGGAAGCA AAGTTTTCA ACGGTTTGGAG ACCGTCCGCC GTAGGCATGC TTTGAGCGT	6240
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CAGCATATCT CCTCGTTTCG CGGGTTGGGG CGGCTTCGC TGTACGGCAG TAGTCGGTGC	6360
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ACCATGGTGT CATAGTCCAG CCCCTCCGCCG CGGTGGCCCT TGGCGCGCAG CTTGCCCTTG	6600
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ATGCGTTTCT TACCTCTGGT TTCCATGAGC CGGTGTCCAC GCTCGGTGAC GAAAAGGCTG	6840

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CGCATGATCG TGCTCCTGTC GTTGAGGACC CGGCTAGGCT GGCGGGGTTG CCTTACTGGT	7740
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GCGACCTGAG CAACAAACATG AATGGTCTTC GGTTCCCGTG TTTCGTAAAG TCTGGAAACG	7860
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GCCCCACCGC TGCCTTAT CGGTAACTA TCGTCTTGAG TCCAACCCGG TAAGACACGA	8100
CTTATCGCCA CTGGCAGCAG CCACTGGTAA CAGGATTAGC AGAGCGAGGT ATGTAGGCGG	8160
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TATCTGCGCT CTGCTGAAGC CAGTTACCTT CGGAAAAAGA GTTGGTAGCT CTTGATCCGG	8280
CAAAACAAACC ACCGCTGGTA GCGGTGGTT TTTTGTTCG AAGCAGCAGA TTACGGCGAG	8340
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CGAAAACCTCA CGTTAAGGGA TTTTGGTCAT GAGATTATCA AAAAGGATCT TCACCTAGAT	8460
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ATCCATAGTT GCCTGACTCC CCGTCGTGTA GATAACTACG ATACGGGAGG GCTTACCATC	8640
TGGCCCCAGT GCTGCAATGA TACCGCGAGA CCCACGCTCA CGGGCTCCAG ATTTATCAGC	8700

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AATAAACCAAG CCAGCCGGAA GGGCCGAGCG CAGAAGTGGT CCTGCAACTT TATCCGCCTC	8760
CATCCAGTCT ATTAATTGTT GCCGGGAGC TAGAGTAAGT AGTTGCCAG TTAATAGTTT	8820
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ATCACTCATG GTTATGGCAG CACTGCATAA TTCTCTTACT GTCATGCCAT CCGTAAGATG	9060
CTTTCTGTG ACTGGTGAGT ACTCAACCAA GTCATTCTGA GAATAGTGTG TCCGGCGACC	9120
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GGCGACACGG AAATGTTGAA TACTCATACT CTTCTTTTT CAATATTATT GAACCATTTA	9420
TCAGGGTTAT TGTCTCATGA GCGGATACAT ATTTGAATGT ATTTAGAAAA ATAAACAAAT	9480
AGGGGTTCCG CGCACATTTC CCCGAAAAGT GCCACCTGAC GTCTAAGAAA CCATTATTAT	9540
CATGACATTA ACCTATAAAA ATAGGCGTAT CACGAGGCC CTTCGTCTTC AA	9592

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 14 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TAGTAATTT GGGC

14

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 14 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGTAAGATTT GGCC

14

75

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGTGAAATCT GAAT

14

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GAATAAATTTT GTGT

14

(2) INFORMATION FOR SEQ ID NC:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGTAATATTT GTCT

14

WHAT IS CLAIMED IS:

1. A recombinant viral vector comprising:
(a) the DNA of, or corresponding to, at least a portion of the genome of an adenovirus, which portion is capable of infecting a hepatic cell;
(b) a human VLDL receptor gene operatively linked to regulatory sequences directing its expression, said gene flanked by the DNA of (a) and capable of expression in the hepatic cell.

2. The vector according to claim 1 wherein said adenovirus DNA comprises the adenovirus 5' and 3' cis-elements necessary for replication and virion encapsidation in the absence of sequence encoding viral genes.

3. The vector according to claim 1 or 2 wherein said adenovirus genome has a deletion in all or a part of the E1 gene.

4. The vector according to any of claims 1 to 3 wherein said adenovirus genome has a deletion in all or a part of the E3 gene.

5. The vector according to any of claims 1 to 3 wherein said adenovirus genome comprising deletions in the DNA sequences of all or a portion of the adenovirus genes selected from the group consisting of the E2a gene, the E4 gene, the late genes L1 through L5, the intermediate genes IX and IV, and a combination thereof.

6. A mammalian hepatocyte which expresses a human VLDL receptor gene introduced therein through transduction of the vector of any one of claims 1 to 5.

7. Use of a recombinant viral vector for the manufacture of a medicament, said viral vector comprising:

(a) the DNA of, or corresponding to, at least a portion of the genome of an adenovirus, which portion is capable of infecting a hepatic cell;

(b) a human VLDL receptor gene operatively linked to regulatory sequences directing its expression, said gene flanked by the DNA of (a) and capable of expression in the hepatic cell.

8. The use according to claim 7 wherein the medicament is used for reducing cholesterol levels in familial hypercholesterolemia patients.

9. The use according to claim 7 wherein the medicament is used for reducing cholesterol levels in familial combined hyperlipidemia patients.

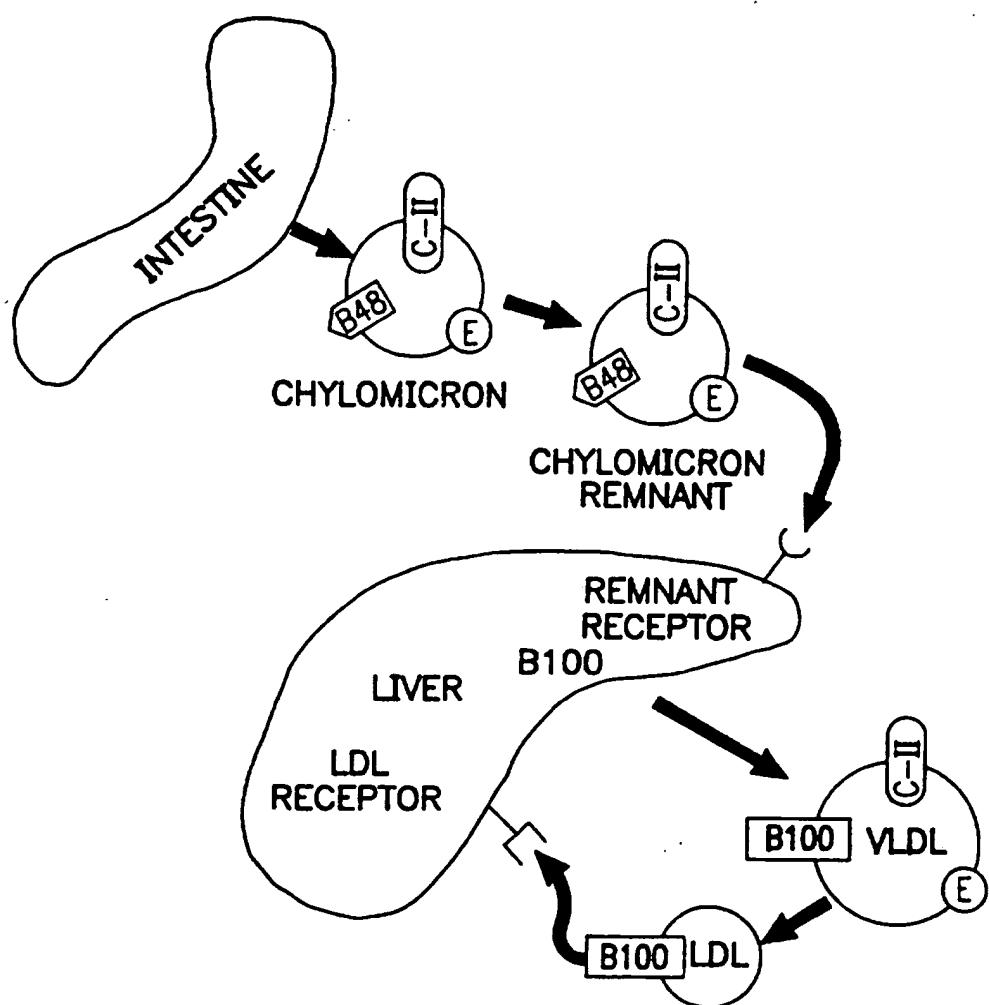


FIG. IA

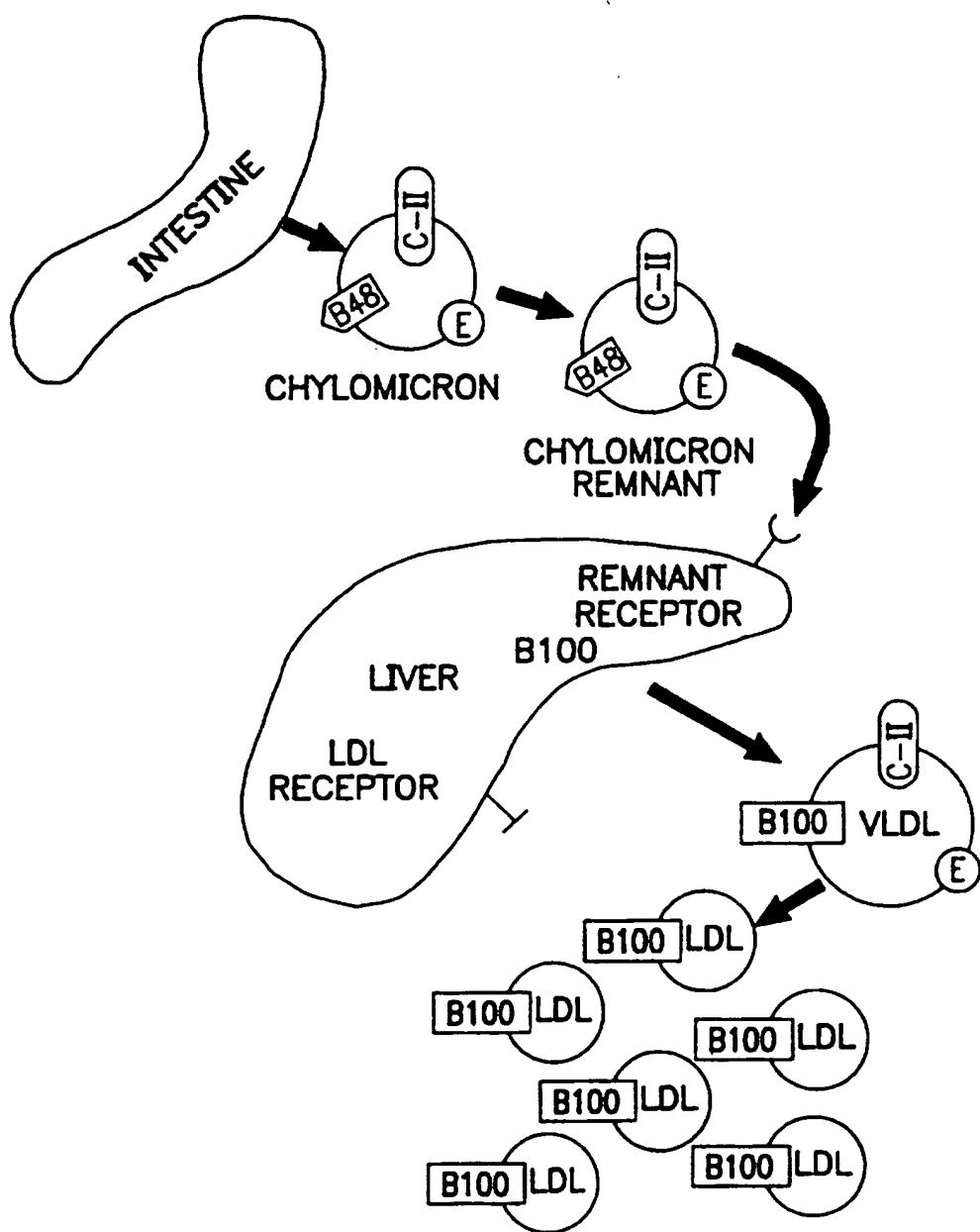


FIG. 1B

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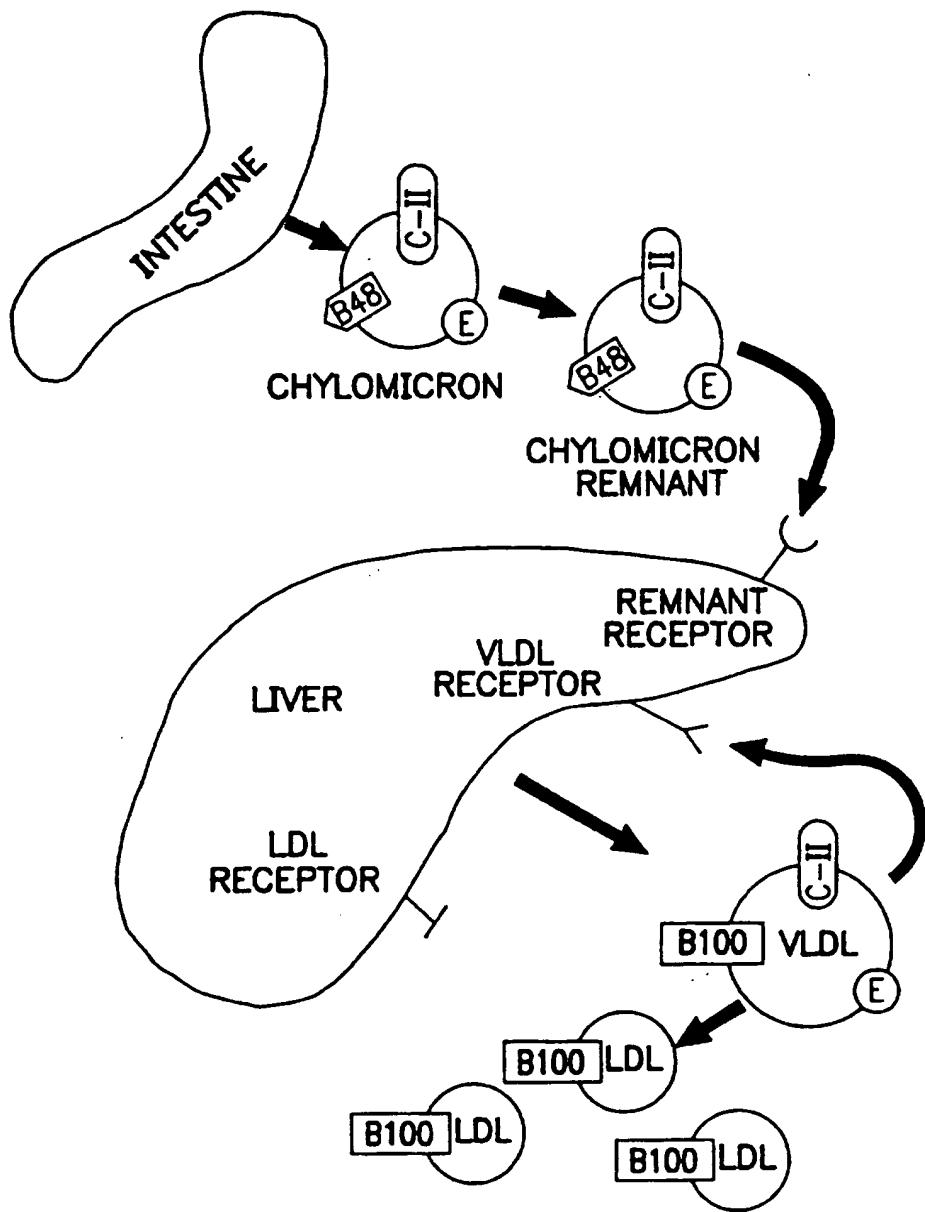


FIG. IC

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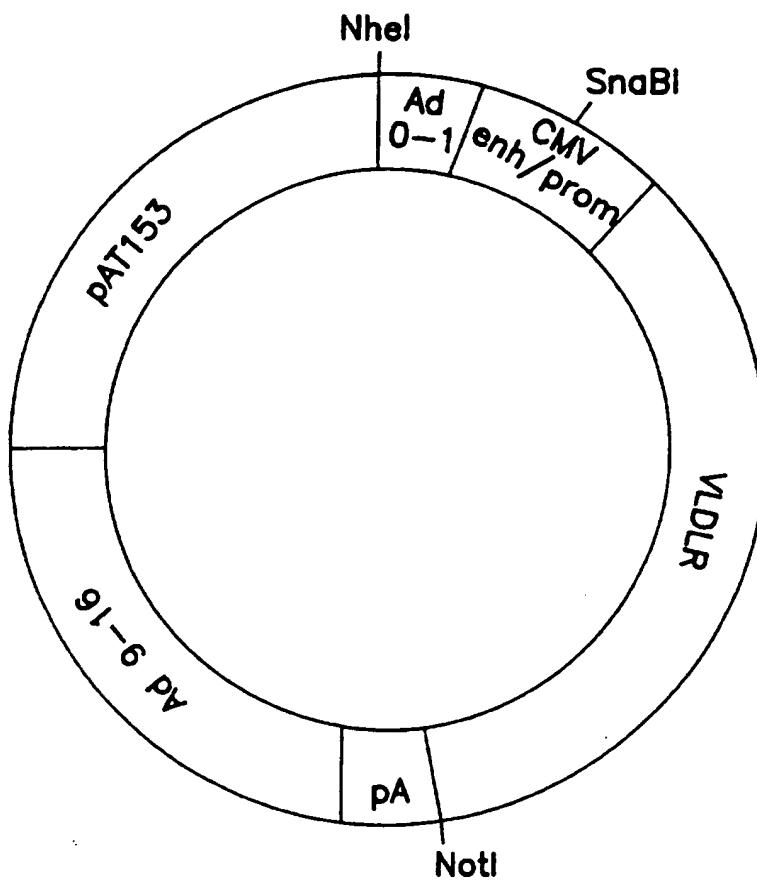


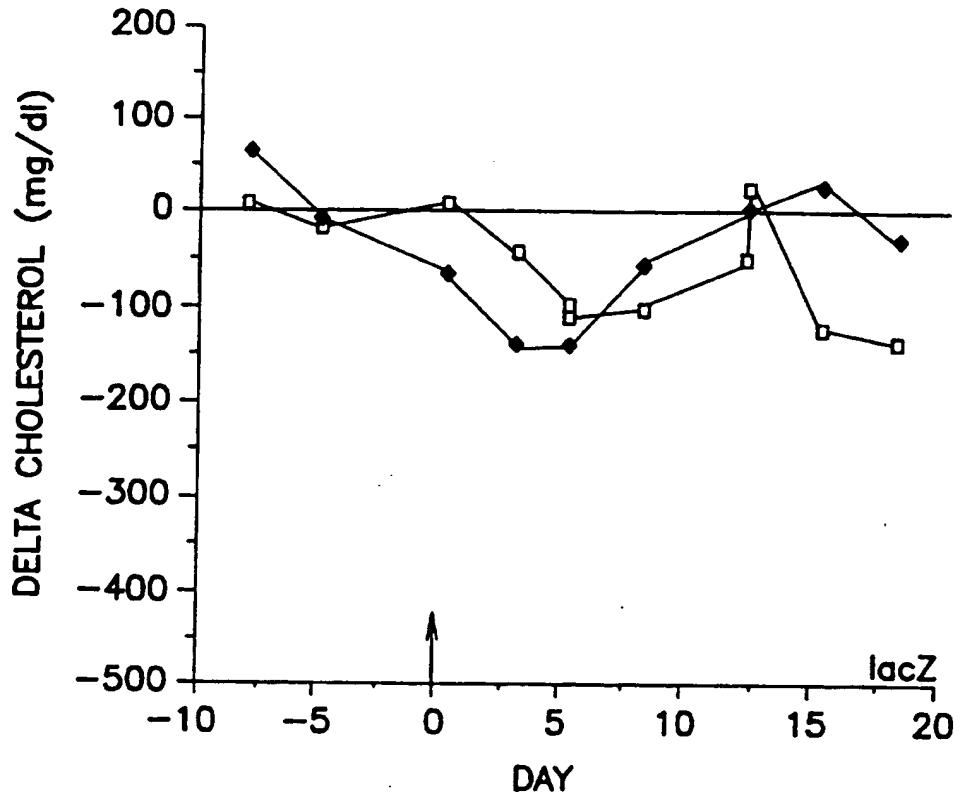
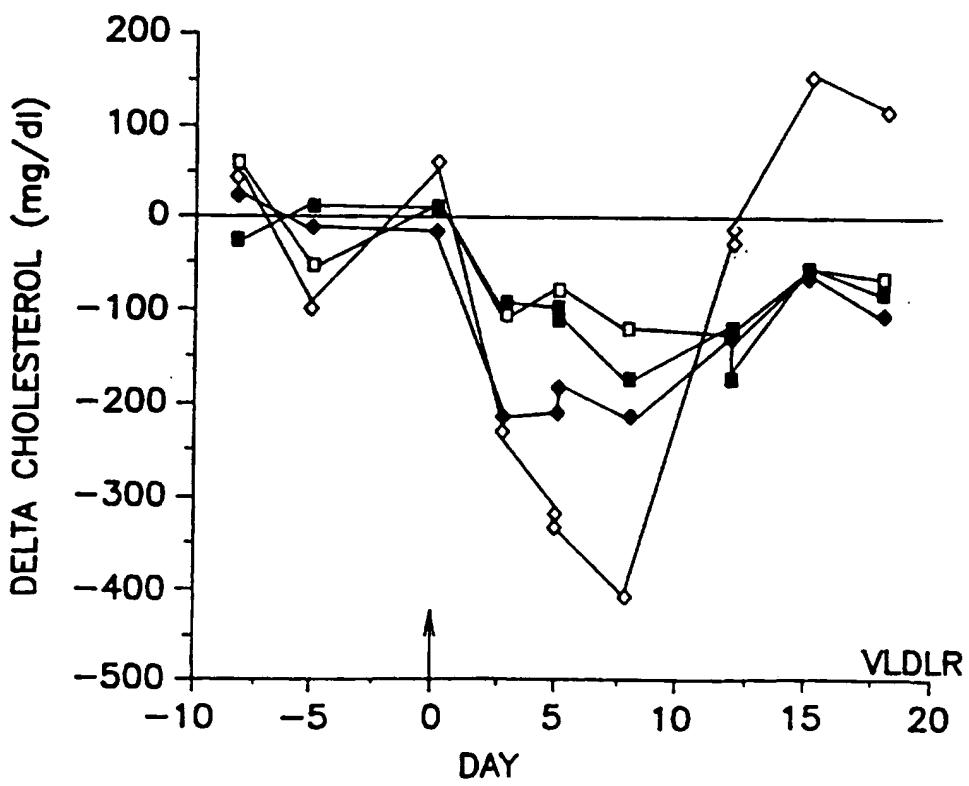
FIG. 2

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FIG. 3

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**FIG. 4A****FIG. 4B**

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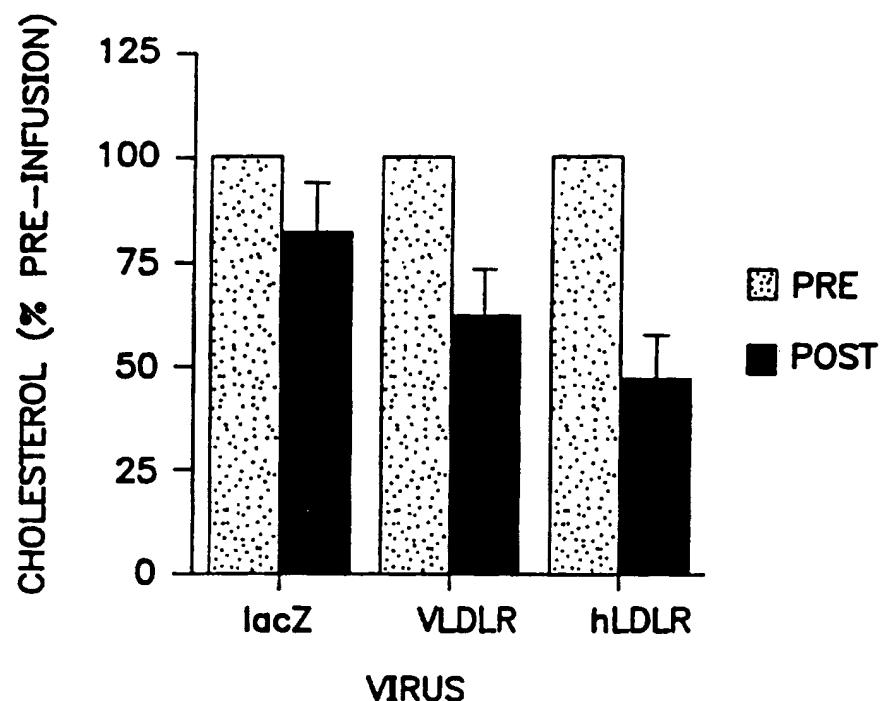


FIG. 5

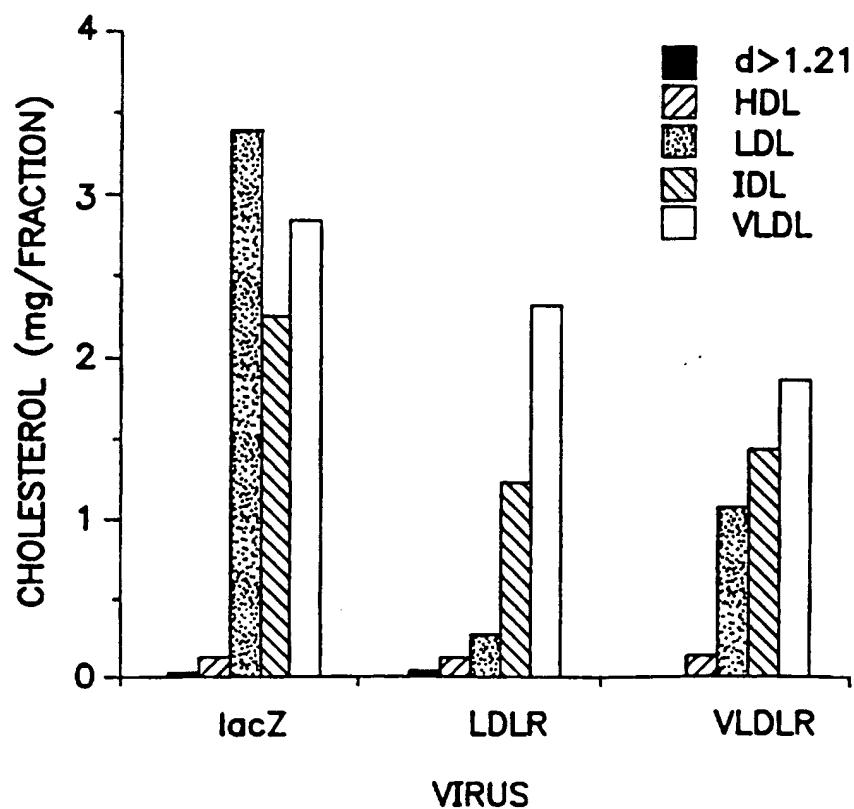


FIG. 6

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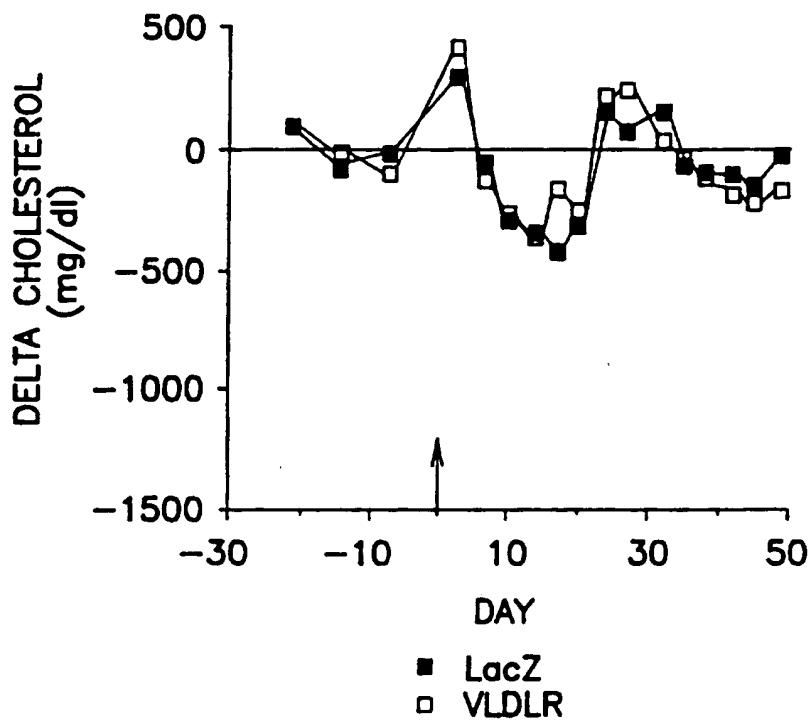


FIG. 7A

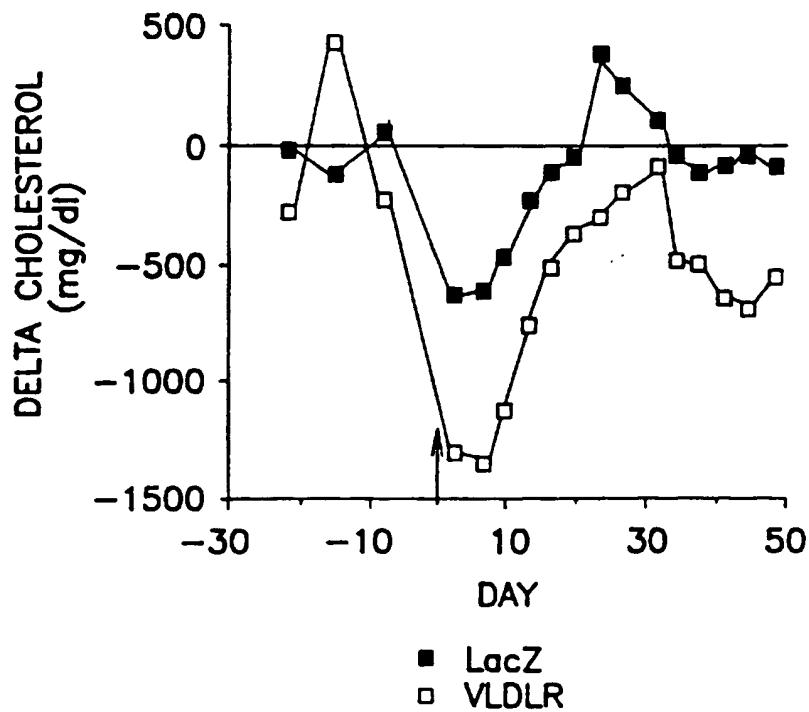


FIG. 7B

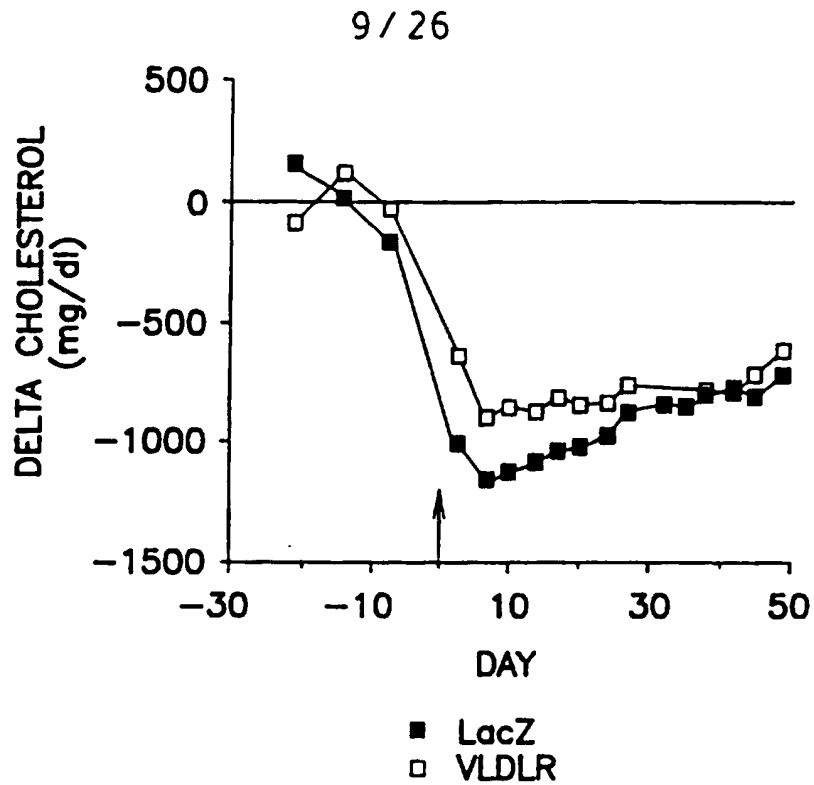


FIG. 7C

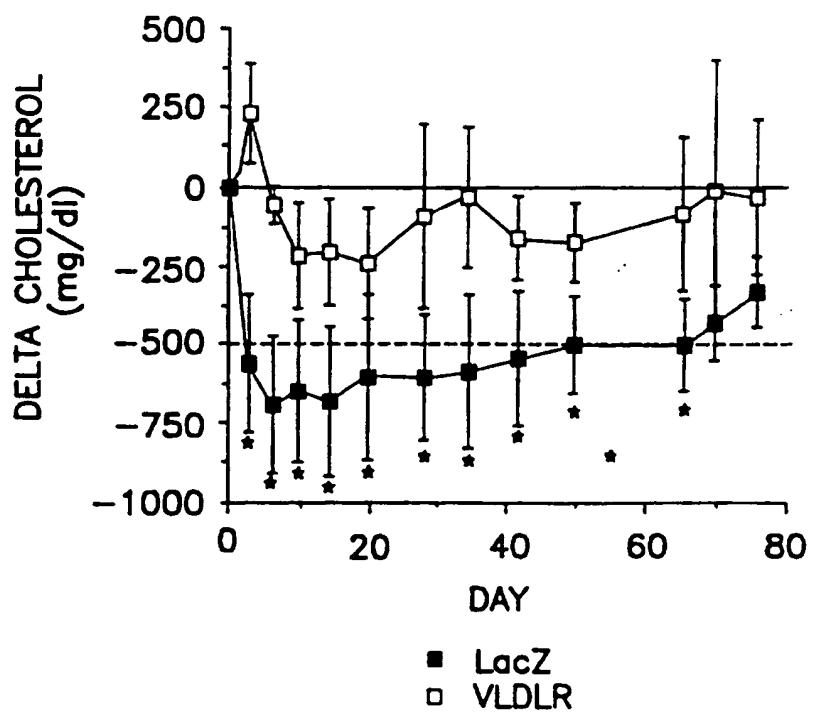


FIG. 7D

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FIGURE 8A

CTCTGCGGGC CGCGGGTGCG GGTGTCGCT ACCGGCTCTC TCCGTTCTGT	50
GCTCTCTTCT GCTCTCGGCT CCCCACCCCC TCTCCCTTCC CTCCCTCTCCC	100
CTTGCCCTCCC CTCCCTTGCA GCGCCTGCAT TATTTTCTGC CCGCAGCTCG	150
GCTTGCACTG CTGCTGCAGC CGGGGGAGGT GGCTGGGTGG GTGGGGAGGA	200
GACTGTGCAA GTTGTAGGGG AGGGGGTGCC CTCTTCTTCC CCGCTCCCTT	250
CCCCAGCCAA GTGGTTCCCC TCCTTCTCCC CCTTTCCCTT CCCAGCCCCC	300
ACCTTCTTCC TCTTTCGGAA GGGCTGGTAA CTTGTCGTGC GGAGCGAACG	350
CGGGCGGCCGG CGGCAGCGGC GGCACCATCC AGGCAGGGCAC C ATG GGC ACG Met Gly Thr	400
	1
TCC GCG CTC TGG GCC GTC TGG CTG CTG CTC GCG CTG TGC TGG	442
Ser Ala Leu Trp Ala Val Trp Leu Leu Leu Ala Leu Cys Trp	
5 10 15	
GCG CCC CGG GAG AGC GGC GCC ACC GGA ACC GGG AGA AAA GCC	484
Ala Pro Arg Glu Ser Gly Ala Thr Gly Thr Gly Arg Lys Ala	
20 25 30	
AAA TGT GAA CCC TCC CAA TTC CAG TGC ACA AAT GGT CGC TGT	526
Lys Cys Glu Pro Ser Gln Phe Gln Cys Thr Asn Gly Arg Cys	
35 40 45	
ATT ACG CTG TTG TGG AAA TGT GAT GGG GAT GAA GAC TGT GTT	568
Ile Thr Leu Leu Trp Lys Cys Asp Gly Asp Glu Asp Cys Val	
50 55	
GAC GGC AGT GAT GAA AAG AAC TGT GTA AAG AAG ACG TGT GCT	610
Asp Gly Ser Asp Glu Lys Asn Cys Val Lys Lys Thr Cys Ala	
60 65 70	
GAA TCT GAC TTC GTG TGC AAC AAT GGC CAG TGT GTT CCC AGC	652
Glu Ser Asp Phe Val Cys Asn Asn Gly Gln Cys Val Pro Ser	
75 80 85	
CGA TGG AAG TGT GAT GGA GAT CCT GAC TGC GAA GAT GGT TCA	694
Arg Trp Lys Cys Asp Gly Asp Pro Asp Cys Glu Asp Gly Ser	
90 95 100	
GAT GAA AGC CCA GAA CAG TGC CAT ATG AGA ACA TGC CGC ATA	736
Asp Glu Ser Pro Glu Gln Cys His Met Arg Thr Cys Arg Ile	
105 110 115	
CAT GAA ATC AGC TGT GGC GCC CAT TCT ACT CAG TGT ATC CCA	778
His Glu Ile Ser Cys Gly Ala His Ser Thr Gln Cys Ile Pro	
120 125	

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FIGURE 8B

GTG TCC TGG AGA TGT GAT GGT GAA AAT GAT TGT GAC AGT GGA Val Ser Trp Arg Cys Asp Gly Glu Asn Asp Cys Asp Ser Gly 130 135 140	820
GAA GAT GAA GAA AAC TGT GGC AAT ATA ACA TGT AGT CCC GAC Glu Asp Glu Glu Asn Cys Gly Asn Ile Thr Cys Ser Pro Asp 145 150 155	862
GAG TTC ACC TGC TCC AGT GGC CGC TGC ATC TCC AGG AAC TTT Glu Phe Thr Cys Ser Ser Gly Arg Cys Ile Ser Arg Asn Phe 160 165 170	904
GTA TGC AAT GGC CAG GAT GAC TGC AGC GAT GGC AGT GAT GAG Val Cys Asn Gly Gln Asp Asp Cys Ser Asp Gly Ser Asp Glu 175 180 185	946
CTG GAC TGT GCC CCG CCA ACC TGT GGC GCC CAT GAG TTC CAG Leu Asp Cys Ala Pro Pro Thr Cys Gly Ala His Glu Phe Gln 190 195	988
TGC AGC ACC TCC TCC TGC ATC CCC ATC AGC TGG GTA TGC GAC Cys Ser Thr Ser Ser Cys Ile Pro Ile Ser Trp Val Cys Asp 200 205 210	1030
GAT GAT GCA GAC TGC TCC GAC CAA TCT GAT GAG TCC CTG GAG Asp Asp Ala Asp Cys Ser Asp Gln Ser Asp Glu Ser Leu Glu 215 220 225	1072
CAG TGT GGC CGT CAG CCA GTC ATA CAC ACC AAG TGT CCA GCC Gln Cys Gly Arg Gln Pro Val Ile His Thr Lys Cys Pro Ala 230 235 240	1114
AGC GAA ATC CAG TGC GGC TCT GGC GAG TGC ATC CAT AAG AAG Ser Glu Ile Gln Cys Gly Ser Gly Glu Cys Ile His Lys Lys 245 250 255	1156
TGG CGA TGT GAT GGG GAC CCT GAC TGC AAG GAT GGC AGT GAT Trp Arg Cys Asp Gly Asp Pro Asp Cys Lys Asp Gly Ser Asp 260 265	1198
GAG GTC AAC TGT CCC TCT CGA ACT TGC CGA CCT GAC CAA TTT Glu Val Asn Cys Pro Ser Arg Thr Cys Arg Pro Asp Gln Phe 270 275 280	1240
GAA TGT GAG GAT GGC AGC TGC ATC CAT GGC AGC AGG CAG TGT Glu Cys Glu Asp Gly Ser Cys Ile His Gly Ser Arg Gln Cys 285 290 295	1282
AAT GGT ATC CGA GAC TGT GTC GAT GGT TCC GAT GAA GTC AAC Asn Gly Ile Arg Asp Cys Val Asp Gly Ser Asp Glu Val Asn 300 305 310	1324

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FIGURE 8C

TGC AAA AAT GTC AAT CAG TGC TTG GGC CCT GGA AAA TTC AAG Cys Lys Asn Val Asn Gln Cys Leu Gly Pro Gly Lys Phe Lys 315 320 325	1366
TGC AGA AGT GGA GAA TGC ATA GAT ATC AGC AAA GTA TGT AAC Cys Arg Ser Gly Glu Cys Ile Asp Ile Ser Lys Val Cys Asn 330 335	1408
CAG GAG CAG GAC TGC AGG GAC TGG AGT GAT GAG CCC CTG AAA Gln Glu Gln Asp Cys Arg Asp Trp Ser Asp Glu Pro Leu Lys 340 345 350	1450
GAG TGT CAT ATA AAC GAA TGC TTG GTA AAT AAT GGT GGA TGT Glu Cys His Ile Asn Glu Cys Leu Val Asn Asn Gly Gly Cys 355 360 365	1492
TCT CAT ATC TGC AAA GAC CTA GTT ATA GGC TAC GAG TGT GAC Ser His Ile Cys Lys Asp Leu Val Ile Gly Tyr Glu Cys Asp 370 375 380	1534
TGT GCA GCT GGG TTT GAA CTG ATA GAT AGG AAA ACC TGT GGA Cys Ala Ala Gly Phe Glu Leu Ile Asp Arg Lys Thr Cys Gly 385 390 395	1576
GAT ATT GAT GAA TGC CAA AAT CCA GGA ATC TGC AGT CAA ATT Asp Ile Asp Glu Cys Gln Asn Pro Gly Ile Cys Ser Gln Ile 400 405	1618
TGT ATC AAC TTA AAA GGC GGT TAC AAG TGT GAA TGT AGT CGT Cys Ile Asn Leu Lys Gly Gly Tyr Lys Cys Glu Cys Ser Arg 410 415 420	1660
GCC TAT CAA ATG GAT CTT GCT ACT GGC GTG TGC AAG GCA GTA Ala Tyr Gln Met Asp Leu Ala Thr Gly Val Cys Lys Ala Val 425 430 435	1702
GGC AAA GAG CCA AGT CTG ATC TTC ACT AAT CGA AGA GAC ATC Gly Lys Glu Pro Ser Leu Ile Phe Thr Asn Arg Arg Asp Ile 440 445 450	1744
AGG AAG ATT GGC TTA GAG AGG AAA GAA TAT ATC CAA CTA GTT Arg Lys Ile Gly Leu Glu Arg Lys Glu Tyr Ile Gln Leu Val 455 460 465	1786
GAA CAG CTA AGA AAC ACT GTG GCT CTC GAT GCT GAC ATT GCT Glu Gln Leu Arg Asn Thr Val Ala Leu Asp Ala Asp Ile Ala 470 475	1828
GCC CAG AAA CTA TTC TGG GCC GAT CTA AGC CAA AAG GCT ATC Ala Gln Lys Leu Phe Trp Ala Asp Leu Ser Gln Lys Ala Ile 480 485 490	1870

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FIGURE 8D

TTC AGT GCC TCA ATT GAT GAC AAG GTT GGT AGA CAT GTT AAA Phe Ser Ala Ser Ile Asp Asp Lys Val Gly Arg His Val Lys 495 500 505	1912
ATG ATC GAC AAT GTC TAT AAT CCT GCA GCC ATT GCT GTT GAT Met Ile Asp Asn Val Tyr Asn Pro Ala Ala Ile Ala Val Asp 510 515 520	1954
TGG GTG TAC AAG ACC ATC TAC TGG ACT GAT GCG GCT TCT AAG Trp Val Tyr Lys Thr Ile Tyr Trp Thr Asp Ala Ala Ser Lys 525 530 535	1996
ACT ATT TCA GTA GCT ACC CTA GAT GGA ACC AAG AGG AAG TTC Thr Ile Ser Val Ala Thr Leu Asp Gly Thr Lys Arg Lys Phe 540 545	2038
CTG TTT AAC TCT GAC TTG CGA GAG CCT GCC TCC ATA GCT GTG Leu Phe Asn Ser Asp Leu Arg Glu Pro Ala Ser Ile Ala Val 550 555 560	2080
GAC CCA CTG TCT GGC TTT GTT TAC TGG TCA GAC TGG GGT GAA Asp Pro Leu Ser Gly Phe Val Tyr Trp Ser Asp Trp Gly Glu 565 570 575	2122
CCA GCT AAA ATA GAA AAA GCA GGA ATG AAT GGA TTC GAT AGA Pro Ala Lys Ile Glu Lys Ala Gly Met Asn Gly Phe Asp Arg 580 585 590	2164
CGT CCA CTG GTG ACA GCG GAT ATC CAG TGG CCT AAC GGA ATT Arg Pro Leu Val Thr Ala Asp Ile Gln Trp Pro Asn Gly Ile 595 600 605	2206
ACA CTT GAC CTT ATA AAA AGT CGC CTC TAT TGG CTT GAT TCT Thr Leu Asp Leu Ile Lys Ser Arg Leu Tyr Trp Leu Asp Ser 610 615	2248
AAG TTG CAC ATG TTA TCC AGC GTG GAC TTG AAT GGC CAA GAT Lys Leu His Met Leu Ser Ser Val Asp Leu Asn Gly Gln Asp 620 625 630	2290
CGT AGG ATA GTA CTA AAG TCT CTG GAG TTC CTA GCT CAT CCT Arg Arg Ile Val Leu Lys Ser Leu Glu Phe Leu Ala His Pro 635 640 645	2332
CTT GCA CTA ACA ATA TTT GAG GAT CGT GTC TAC TGG ATA GAT Leu Ala Leu Thr Ile Phe Glu Asp Arg Val Tyr Trp Ile Asp 650 655 660	2374
GGG GAA AAT GAA GCA GTC TAT GGT GCC AAT AAA TTC ACT GGA Gly Glu Asn Glu Ala Val Tyr Gly Ala Asn Lys Phe Thr Gly 665 670 675	2416

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FIGURE 8E

TCA GAG CAT GCC ACT CTA GTC AAC AAC CTG AAT GAT GCC CAA Ser Glu His Ala Thr Leu Val Asn Asn Leu Asn Asp Ala Gln 680 685	2458
GAC ATC ATT GTC TAT CAT GAA CTT GTA CAG CCA TCA GGT AAA Asp Ile Ile Val Tyr His Glu Leu Val Gln Pro Ser Gly Lys 690 695 700	2500
AAT TGG TGT GAA GAA GAC ATG GAG AAT GGA GGA TGT GAA TAC Asn Trp Cys Glu Glu Asp Met Glu Asn Gly Gly Cys Glu Tyr 705 710 715	2545
CTA TGC CTG CCA GCA CCA CAG ATT AAT GAT CAC TCT CCA AAA Leu Cys Leu Pro Ala Pro Gln Ile Asn Asp His Ser Pro Lys 720 725 730	2584
TAT ACC TGT TCC TGT CCC AGT GGG TAC AAT GTA GAG GAA AAT Tyr Thr Cys Ser Cys Pro Ser Gly Tyr Asn Val Glu Glu Asn 735 740 745	2626
GGC CGA GAC TGT CAA AGT ACT GCA ACT ACT GTG ACT TAC AGT Gly Arg Asp Cys Gln Ser Thr Ala Thr Thr Val Thr Tyr Ser 750 755	2668
GAG ACA AAA GAT ACG AAC ACA ACA GAA ATT TCA GCA ACT AGT Glu Thr Lys Asp Thr Asn Thr Glu Ile Ser Ala Thr Ser 760 765 770	2710
GGA CTA GTT CCT GGA GGG ATC AAT GTG ACC ACA GCA GTA TCA Gly Leu Val Pro Gly Gly Ile Asn Val Thr Thr Ala Val Ser 775 780 785	2752
GAG GTC AGT GTT CCC CCA AAA GGG ACT TCT GCC GCA TGG GCC Glu Val Ser Val Pro Pro Lys Gly Thr Ser Ala Ala Trp Ala 790 795 800	2794
ATT CTT CCT CTC TTG CTC TTA GTG ATG GCA GCA GTA GGT GGC Ile Leu Pro Leu Leu Leu Val Met Ala Ala Val Gly Gly 805 810 815	2836
TAC TTG ATG TGG CCG AAT TGG CAA CAC AAG AAC ATG AAA AGC Tyr Leu Met Trp Arg Asn Trp Gln His Lys Asn Met Lys Ser 820 825	2878
ATG AAC TTT GAC AAT CCT GTG TAC TTG AAA ACC ACT GAA GAG Met Asn Phe Asp Asn Pro Val Tyr Leu Lys Thr Thr Glu Glu 830 835 840	2920
GAC CTC TCC ATA GAC ATT GGT AGA CAC AGT GCT TCT GTT GGA Asp Leu Ser Ile Asp Ile Gly Arg His Ser Ala Ser Val Gly 845 850 855	2962

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FIGURE 8F

CAC ACG TAC CCA GCA ATA TCA GTT GTA AGC ACA GAT GAT GAT	3004
His Thr Tyr Pro Ala Ile Ser Val Val Ser Thr Asp Asp Asp	
860 865 870	
CTA GCT TGACTTCTGT GACAAATGTT GACCTTGAG GTCTAACAA	3050
Leu Ala	
ATAATACCCC CGTCGGAATG GTAAACCGAGC CAGCAGCTGA AGTCTCTTTT	3100
TCTTCCTCTC GGCTGGAAAGA ACATCAAGAT ACCTTTGCGT GGATCAAGCT	3150
TGCTGTACTT GACCGTTTTT ATATTACTTT TGTAATATT CTTGTCCACA	3200
TTCTACTTCA GCTTTGGATG TGGTTACCGA GTATCTGTAA CCCTTGAATT	3250
TCTAGACAGT ATTGCCACCT CTGGCCAAAT ATGCACTTTC CCTAGAAAGC	3300
CATATTCCAG CAGTGAAACT TGTGCTATAG TGTATACCAC CTGTACATAC	3350
ATTGTATAGG CCATCTGTAA ATATCCCAGA GAACAATCAC TATTCTTAAG	3400
CACTTTGAAA ATATTTCTAT GTAAATTATT GTAAACTTT TCAATGGTTG	3450
GGACAATGGC AATAGGACAA AACGGGTTAC TAAGATGAAA TTGCCAAAAA	3500
AATTTATAAA CTAATTTGG TACGTATGAA TGATATCTTT GACCTCAATG	3550
GAGGTTTGCA AAGACTGAGT GTTCAAACTA CTGTACATTT TTTTTCAAGT	3600
GCTAAAAAAT TAAACCAAGC AGCTTAAAAA AAAAAAAAAA AAAAAAAAAA	3650
AAAAAA	3656

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FIGURE 9A

GAATTCGCTA GCATCATCAA TAATATAACCT TATTTGGAT TGAAGCCAAT ATGATAATGA
60
GGGGGTGGAG TTTGTGACGT GGCGCGGGC GTGGGAACGG GGCGGGTGAC GTAGTAGTGT
120
GGCGGAAGTG TGATGTTGCA AGTGTGGCGG AACACATGTA AGCGACGGAT GTGGCAAAAG
180
TGACGTTTT GGTGTGCGCC GGTGTACACA GGAAGTGACA ATTTTCGCGC GGTTTTAGGC
240
GGATGTTGTA GTAAATTGG GCGTAACCGA GTAAGATTG GCCATTTCG CGGGAAAACT
300
GAATAAGAGG AAGTGAAATC TGAATAATT TGTGTTACTC ATAGCGCGTA ATATTGCT
360
AGGGAGATCA GCCTGCAGGT CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG
420
CCCAACGACC CCCGCCATT GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA
480
GGGACTTTCC ATTGACGTCA ATGGGTGGAG TATTACGGT AAAC TGCCCA CTTGGCAGTA
540
CATCAA GTGT ATCATATGCC AAGTACGCC CCTATTGACG TCAATGACGG TAAATGGCCC
600
GCCTGGCATT ATGCCAGTA CATGACCTA TGGGACTTTC CTACTGGCA GTACATCTAC
660
GTATTAGTCA TCGCTATTAC CATGGTGATG CGGTTTGGC AGTACATCAA TGGCGTGG
720
TAGCGGTTTG ACTCACGGGG ATTTCCAAGT CTCCACCCCCA TTGACGTCAA TGGGAGTTG
780
TTTTGGCACC AAAATCAACG GGACTTTCCA AAATGTCGT ACAACTCCGC CCCATTGACG
840
CAAATGGCG GTAGGCGTGT ACGGTGGGAG GTCTATATAA GCAGAGCTCT CTGGCTAACT
900
AGAGAACCCA CTGCTTAACG GGCTTATCGA AATTAATACG ACTCACTATA GGGAGACCCA
960
AGCTTCTCTG CGGGCCGCGG GTGCGGGTCG TCGCTACCGG CTCTCTCCGT TCTGTGCTCT
1020
CTTCTGCTCT CGGCTCCCCA CCCCCCTCTCC CTTCCCTCCT CTCCCCCTTGC CTCCCCCTCCT
1080

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FIGURE 9B

CTGCAGCGCC TGCATTATTT TCTGCCCGCA GCTCGGCTTG CACTGCTGCT GCAGCCCCGGG
1140
GAGGTGGCTG GGTGGGTGGG GAGGAGACTG TGCAAGTTGT AGGGGAGGGG GTGCCCTCTT
1200
CTTCCCCGCT CCCTTCCCCA GCCAAGTGGT TCCCCTCCTT CTCCCCCTTT CCCCTCCCAG
1260
CCCCCACCTT CTTCTCTTT CGGAAGGGCT GGTAACCTGT CGTGCAGGAGC GAACGGCGGC
1320
GGCGGCGGCG CGGGCGGCAC CATCCAGGCG GGCACCATGG GCACGTCCGC GCTCTGGGCC
1380
GTCTGGCTGC TGCTCGCGCT GTGCTGGCG CCCCCGGAGA GCGGCGCCAC CGGAACCGGG
1440
AGAAAAGCCA AATGTGAACC CTCCCAATTG CAGTGCACAA ATGGTCGCTG TATTACGCTG
1500
TTGTGGAAAT GTGATGGGGA TGAAGACTGT GTTGACGGCA GTGATGAAAA GAACTGTGTA
1560
AAGAAGACGT GTGCTGAATC TGACTTCGTG TGCAACAATG GCCAGTGTGT TCCCAGCCGA
1620
TGGAAAGTGTG ATGGAGATCC TGACTGCGAA GATGGTTTAG ATGAAAGCCC AGAACAGTGC
1680
CATATGAGAA CATGCCGCAT ACATGAAATC AGCTGTGGCG CCCATTCTAC TCAGTGTATC
1740
CCAGTGTCCCT GGAGATGTGA TGGTGAAAAT GATTGTGACA GTGGAGAAGA TGAAGAAAAC
1800
TGTGGCAATA TAACATGTAG TCCCGACGAG TTCACCTGCT CCAGTGGCCG CTGCATCTCC
1860
AGGAACTTG TATGCAATGG CCAGGATGAC TGCAGCGATG GCAGTGATGA GCTGGACTGT
1920
GCCCGCCAA CCTGTGGCGC CCATGAGTTC CAGTGCAGCA CCTCCTCCTG CATCCCCATC
1980
AGCTGGGTAT GCGACGATGA TGCAGACTGC TCCGACCAAAT CTGATGAGTC CCTGGAGCAG
2040
TGTGGCCGTC AGCCAGTCAT ACACACCAAG TGTCCAGCCA GCGAAATCCA GTGCGGCTCT
2100
GGCGAGTGCA TCCATAAGAA GTGGCGATGT GATGGGGACC CTGACTGCAA GGATGGCAGT
2160

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FIGURE 9C

GATGAGGTCA ACTGTCCCTC TCGAACTTGC CGACCTGACC AATTTGAATG TGAGGGATGGC
2220
AGCTGCATCC ATGGCAGCAG GCAGTGTAAT GGTATCCGAG ACTGTGTCGA TGGTTCCGAT
2280
GAAGTCAACT GCAAAAATGT CAATCAGTGC TTGGGCCCTG GAAAATTCAA GTGCAGAAGT
2340
GGAGAATGCA TAGATATCAG CAAAGTATGT AACCAAGGAGC AGGACTGCAG GGACTGGAGT
2400
GATGAGCCCC TGAAAGAGTG TCATATAAAC GAATGCTTGG TAAATAATGG TGGATGTTCT
2460
CATATCTGCA AAGACCTAGT TATAGGCTAC GAGTGTGACT GTGCAGCTGG GTTGAACTG
2520
ATAGATAGGA AAACCTGTGG AGATATTGAT GAATGCCAAA ATCCAGGAAT CTGCAGTCAA
2580
ATTTGTATCA ACTTAAAAGG CGGTTACAAG TGTGAATGTA GTCTGCCCTA TCAAATGGAT
2640
CTTGCTACTG GCGTGTGCAA GGCAGTAGGC AAAGAGCCAA GTCTGATCTT CACTAATCGA
2700
AGAGACATCA GGAAGATTGG CTTAGAGAGG AAAGAATATA TCCAACTAGT TGAACAGCTA
2760
AGAAACACTG TGGCTCTCGA TGCTGACATT GCTGCCAGA AACTATTCTG GGCCGATCTA
2820
AGCCAAAAGG CTATCTTCAG TGCCTCAATT GATGACAAGG TTGGTAGACA TGTTAAAATG
2880
ATCGACAATG TCTATAATCC TGCAGCCATT GCTGTTGATT GGGTGTACAA GACCATCTAC
2940
TGGACTGATG CGGCTTCTAA GACTATTCA GTAGCTACCC TAGATGGAAC CAAGAGGAAG
3000
TTCCTGTTTA ACTCTGACTT GCGAGAGCCT GCCTCCATAG CTGTGGACCC ACTGTCTGGC
3060
TTTGTAACT GGTCAGACTG GGGTGAACCA GCTAAAATAG AAAAAGCAGG AATGAATGGA
3120
TTCGATAGAC GTCCACTGGT GACAGCGGAT ATCCAGTGGC CTAACGGAAT TACACTTGAC
3180
CTTATAAAAAA GTCGCCTCTA TTGGCTTGAT TCTAAGTTGC ACATGTTATC CAGCGTGGAC
3240

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FIGURE 9D

TTGAATGGCC AAGATCGTAG GATA GTACTA AAGTCTCTGG AGTCCTAGC TCATCCTCTT
3300
GCACTAACAA TATTTGAGGA TCGTGTCTAC TGGATAGATG GGGAAAATGA AGCAGTCTAT
3360
GGTGCCAATA AATTCACTGG ATCAGAGCAT GCCACTCTAG TCAACAACCT GAATGATGCC
3420
CAAGACATCA TTGTCTATCA TGAACTTGTA CAGCCATCAG GTAAAATTG GTGTGAAGAA
3480
GACATGGAGA ATGGAGGATG TGAATACCTA TGCCCTGCCAG CACCACAGAT TAATGATCAC
3540
TCTCCAAAAT ATACCTGTT CTGTCCCAGT GGGTACAATG TAGAGGAAAA TGGCCGAGAC
3600
TGTCAAAGTA CTGCAACTAC TGTGACTTAG AGACAAAAGA TACGAACACA ACAGAAATT
3660
CAGCAACTAG TGGACTAGTT CCTGGAGGGA TCAATGTGAC CACAGCAGTA TCAGAGGTCA
3720
GTGTTCCCCC AAAAGGGACT TCTGCCGCAT GGGCCATTCT TCCTCTCTTG CTCTTAGTGA
3780
TGGCAGCAGT AGGTGGCTAC TTGATGTGGC GGAATTGGCA ACACAAGAAC ATGAAAAGCA
3840
TGAACTTGA CAATCCTGTG TACTTGAAAA CCACTGAAGA GGACCTCTCC ATAGACATTG
3900
GTAGACACAG TGCTTCTGTT GGACACACGT ACCCAGCAAT ATCAGTTGTA AGCACAGATG
3960
ATGATCTAGC TTGACTTCTG TGACAAATGT TGACCTTGA GGTCTAAACA AATAATACCC
4020
CCGTCGGAAT GGTAACCGAG CCAGCAGCTG AAGTCTCTT TTCTTCCTCT CGGCTGGAAG
4080
AACATCAAGA TACCTTGCG TGGATCAAGC TTGGTACCGA GCTCGGATCC ACTAGTAACG
4140
GCCGCCAGTG TGCTGGAATT CTGCAGATAT CCATCACACT GGCGGCCGCG GGGATCCAGA
4200
CATGATAAGA TACATTGATG AGTTGGACA AACCCACAAGT AGAATGCAGT GAAAAAAATG
4260
CTTTATTGT GAAATTGTG ATGCTATTGC TTTATTGTA ACCATTATAA GCTGCAATAA
4320

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FIGURE 9E

ACAAGTTAAC ACAACAATT GCATTCATTT TATGTTTCAG GTTCAGGGGG AGGTGTGGGA
4380
GGTTTTTCG GATCCTCTAG AGTCGACCTG CAGGCTGATC TGGAAGGTGC TGAGGTACGA
4440
TGAGACCCGC ACCAGGTGCA GACCCTGCGA GTGTGGCGGT AAACATATTA GGAACCAGCC
4500
TGTGATGCTG GATGTGACCG AGGAGCTGAG GCCCGATCAC TTGGTGCTGG CCTGCACCCG
4560
CGCTGAGTT GGCTCTAGCG ATGAAGATAAC AGATTGAGGT ACTGAAATGT GTGGGCGTGG
4620
CTTAAGGGTG GGAAAGAATA TATAAGGTGG GGGTCTTATG TAGTTTGTA TCTGTTTGC
4680
AGCAGCCGCC GCCGCCATGA GCACCAAATC GTTTGATGGA AGCATTGTGA GCTCATATTT
4740
GACAACGCGC ATGCCCCAT GGGCCGGGGT GCGTCAGAAT GTGATGGGCT CCAGCATTGA
4800
TGGTCGCCCG GTCCTGCCCG CAAACTCTAC TACCTTGACC TACGAGACCG TGTCTGGAAC
4860
GCCGTTGGAG ACTGCAGCCT CCGCCGCCGC TTCAGCCGCT GCAGCCACCG CCCGCGGGAT
4920
TGTGACTGAC TTTGCTTTCC TGAGCCCGCT TGCAAGCAGT GCAGCTTCCC GTTCATCCGC
4980
CCGCGATGAC AAGTTGACGG CTCTTTGGC ACAATTGGAT TCTTGACCC GGGAACTTAA
5040
TGTCTTTCT CAGCAGCTGT TGGATCTGCG CCAGCAGGTT TCTGCCCTGA AGGCTTCCTC
5100
CCCTCCCAAT GCGGTTAAA ACATAAATAA AAAACCAGAC TCTGTTGGA TTTGGATCAA
5160
GCAAGTGTCT TGCTGTCTTT ATTTAGGGGT TTTGCGCGCG CGGTAGGCCCG GGGACCAAGCG
5220
GTCTCGGTG TGAGGGTCC TGTGTATTT TTCCAGGACG TGGTAAAGGT GACTCTGGAT
5280
GTTCAAGATAAC ATGGGCATAA GCCCGTCTCT GGGGTGGAGG TAGCACCAC GCAGAGCTTC
5340
ATGCTGCAGGG GTGGTGTGT AGATGATCCA GTCTAGCAG GAGCGCTGGG CGTGGTGCCT
5400

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FIGURE 9F

AAAAATGTCT TTCAGTAGCA AGCTGATTGC CAGGGCAGG CCCTTGGTGT AAGTGTTCAC
5460

AAAGCGGTTA AGCTGGGATG GGTGCATAACG TGGGGATATG AGATGCATCT TGGACTGTAT
5520

TTTTAGGTTG GCTATGTTCC CAGCCATATC CCTCCGGGA TTCAATGTTGT GCAGAACAC
5580

CAGCACAGTG TATCCGGTGC ACTTGGGAAA TTTGTCAATGT AGCTTAGAAG GAAATGCGTG
5640

GAAGAACTTG GAGACGCCCT TGTGACCTCC AAGATTTCC ATGCATTCTG CCATAATGAT
5700

GGCAATGGGC CCACGGCGG CGGCCTGGGC GAAGATATT CTGGGATCAC TAACGTCATA
5760

GTTGTGTTCC AGGATGAGAT CGTCATAGGC CATTTCACA AAGCGCGGGC GGAGGGTGCC
5820

AGACTGCGGT ATAATGGTTC CATCCGGCCC AGGGGCGTAG TTACCCCTCAC AGATTTGCAT
5880

TTCCCCACGCT TTGAGTTCAAG ATGGGGGGAT CATGTCTACC TGCGGGCGA TGAAGAAAAC
5940

GGTTTCCGGG GTAGGGGAGA TCAGCTGGGA AGAAAGCAGG TTCCTGAGCA GCTGCGACTT
6000

ACCGCAGCCG GTGGGCCCGT AAATCACACC TATTACCGGG TGCAACTGGT AGTTAAGAGA
6060

GCTGCAGCTG CCGTCATCCC TGAGCAGGGG GGCCACTTCG TTAAGCATGT CCCTGACTCG
6120

CATGTTTCC CTGACCAAAT CCGCCAGAAG GCGCTCGCCG CCCAGCGATA GCAGTTCTTG
6180

CAAGGAAGCA AAGTTTTCA ACGGTTGAG ACCGTCCGCC GTAGGCATGC TTTTGAGCGT
6240

TTGACCAAGC AGTTCCAGGC GGTCCCACAG CTCGGTCACC TGCTCTACGG CATCTCGATC
6300

CAGCATATCT CCTCGTTTCG CGGGTTGGGG CGGCTTCGC TGTACGGCAG TAGTCGGTGC
6360

TCGTCCAGAC GGGCCAGGGT CATGTCTTC CACGGCGCA GGGTCCTCGT CAGCGTAGTC
6420

TGGGTACGG TGAAGGGGTG CGCTCCGGGC TGCAGCGTGG CCAGGGTGCG CTTGAGGCTG
6480

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FIGURE 9G

GTCCCTGCTGG TGCTGAAGCG CTGCCGGTCT TCGCCCTGCG CGTCGGCCAG GTAGCATTG
6540
ACCATGGTGT CATAGTCCAG CCCCTCCGCG GCGTGGCCCT TGGCGCGCAG CTTGCCCTG
6600
GAGGAGGCAGC CGCACCGAGGG GCAGTGCAGA CTTTGAGGG CGTAGAGCTT GGGCGCGAGA
6660
AATACCGATT CCAGGGAGTA GGCATCCGCG CCGCAGGCC CGCAGACGGT CTCGCATTCC
6720
ACGAGCCAGG TGAGCTCTGG CCGTTGGGG TCAAAAACCA GGTTTCCCCC ATGCTTTTG
6780
ATGCGTTCT TACCTCTGGT TTCCATGAGC CGGTGTCCAC GCTCGGTGAC GAAAAGGCTG
6840
TCCGTGTCCC CGTATACAGA CTTGAGAGGC CTGTCCTCGA CCGATGCCCT TGAGAGCCTT
6900
CAACCCAGTC AGCTCCTTCC GGTGGGCGCG GGGCATGACT ATCGTCGCCG CACTTATGAC
6960
TGTCTTCTTT ATCATGCAAC TCGTAGGACA GGTGCCGGCA GCGCTCTGGG TCATTTTCGG
7020
CGAGGACCGC TTTCGCTGGA GCGCGACGAT GATCGGCCTG TCGCTTGCGG TATTCGGAAT
7080
CTTGCACGCC CTCGCTCAAG CCTTCGTCAC TGGTCCCGCC ACCAACGTT TCGCGAGAA
7140
GCAGGCCATT ATCGCCGGCA TGGCGGCCGA CGCGCTGGC TACGTCTTGC TGGCGTTCGC
7200
GACGCGAGGC TGGATGGCCT TCCCCATTAT GATTCTTCTC GCTTCCGGCG GCATCGGGAT
7260
GCCCGCGTTG CAGGCCATGC TGTCCAGGCA GGTAGATGAC GACCATCAGG GACAGCTTCA
7320
AGGATCGCTC GCGGCTCTTA CCAGCCTAAC TTCGATCACT GGACCGCTGA TCGTCACGGC
7380
GATTTATGCC GCCTCGCGA GCACATGGAA CGGGTTGGCA TGGATTGTAG GCGCCGCCCT
7440
ATACCTTGTC TGCCTCCCCG CGTTGCGTCG CGGTGCATGG AGCCGGGCCA CCTCGACCTG
7500
AATGGAAGCC GGCAGGCACCT CGCTAACGGA TTCACCACTC CAAGAATTGG AGCCAATCAA
7560

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FIGURE 9H

TTCTTGCAGGA GAACTGTGAA TCGCAAAACC AACCCCTGGC AGAACATATC CATCGCGTCC
7620
GCCATCTCCA GCAGCCGCAC CGGGCGCATC TCGGGCAGCG TTGGGTCTTG GCCACGGGTG
7680
CGCATGATCG TGCTCCTGTC GTTGAGGACC CGGCTAGGCT GGCGGGGTG CCTTACTGGT
7740
TAGCAGAATG AATCACCGAT ACGCGAGCGA ACGTGAAGCG ACTGCTGCTG CAAAACGTCT
7800
GCGACCTGAG CAACAACATG AATGGTCTTC GGTTTCCGTG TTTCGTAAAG TCTGGAAACG
7860
CGGAAGTCAG CGCCCTGCAC CATTATGTTT CGGATCTGCA TCGCAGGATG CTGCTGGCTA
7920
CCCTGTGGAA CACCTACATC TGTATTAAACG AAGCCTTTCT CAATGCTCAC GCTGTAGGTA
7980
TCTCAGTTCG GTGTAGGTCG TTGCTCCAA GCTGGGCTGT GTGCACGAAC CCCCCGTTCA
8040
GCCCGACCGC TGCGCCTTAT CCGGTAACTA TCGTCTTGAG TCCAACCCGG TAAGACACGA
8100
CTTATCGCCA CTGGCAGCAG CCACTGGTAA CAGGATTAGC AGAGCGAGGT ATGTAGGCGG
8160
TGCTACAGAG TTCTTGAAGT GGTGGCCTAA CTACGGCTAC ACTAGAAGGA CAGTATTTGG
8220
TATCTGCGCT CTGCTGAAGC CAGTTACCTT CGGAAAAAGA GTTGGTAGCT CTTGATCCGG
8280
CAAACAAACC ACCGCTGGTA GCGGTGGTTT TTTTGTGTC AAGCAGCAGA TTACGCGCAG
8340
AAAAAAAGGA TCTCAAGAAG ATCCTTGAT CTTTCTACG GGGTCTGACG CTCAGTGGAA
8400
CGAAAATCA CGTTAAGGGA TTTTGGTCAT GAGATTATCA AAAAGGATCT TCACCTAGAT
8460
CCTTTAAAT TAAAAATGAA GTTTAAATC AATCTAAAGT ATATATGAGT AAACCTGGTC
8520
TGACAGTTAC CAATGCTTAA TCAGTGAGGC ACCTATCTCA GCGATCTGTC TATTCGTTG
8580
ATCCATAGTT GCCTGACTCC CCGTCGTGTA GATAACTACG ATACGGGAGG GCTTACCATC
8640

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FIGURE 9I

TGGCCCCAGT GCTGCAATGA TACCGCGAGA CCCACGCTCA CCGGCTCCAG ATTTATCAGC
8700

AATAAACAG CCAGCCGGAA GGGCCGAGCG CAGAAGTGGT CCTGCAACTT TATCCGCCTC
8760

CATCCAGTCT ATTAATTGTT GCCGGGAAGC TAGAGTAAGT AGTCGCCAG TTAATAGTTT
8820

GCGAACGTT GTTGCCATTG CTGCAGGCAT CGTGGTGTCA CGCTCGTCGT TTGGTATGGC
8880

TTCATTCAAGC TCCGGTTCCC AACGATCAAG GCGAGTTACA TGATCCCCA TGTTGTGCAA
8940

AAAAGCGGTT AGCTCCTTCG GTCCTCCGAT CGTTGTCAGA AGTAAGTTGG CCGCAGTGT
9000

ATCACTCATG GTTATGGCAG CACTGCATAA TTCTCTTACT GTCATGCCAT CCGTAAGATG
9060

CTTTTCTGTG ACTGGTGAGT ACTCAACCAA GTCATTCTGA GAATAGTGT A TGCGGCGACC
9120

GAGTTGCTCT TGCCCGGCGT CAACACGGGA TAATACCGCG CCACATAGCA GAACTTTAAA
9180

AGTGCTCATC ATTGGAAAAC GTTCTCGGG GCGAAAAC TCAGGATCT TACCGCTGTT
9240

GAGATCCAGT TCGATGTAAC CCACTCGTGC ACCCAACTGA TCTTCAGCAT CTTTTACTTT
9300

CACCAGCGTT TCTGGGTGAG CAAAAACAGG AAGGCAAAT GCGCAAAAA AGGGATAAG
9360

GGCGACACGG AAATGTTGAA TACTCATACT CTTCCCTTTT CAATATTATT GAAGCATTAA
9420

TCAGGGTTAT TGTCTCATGA GCGGATACAT ATTTGAATGT ATTTAGAAAA ATAAACAAAT
9480

AGGGGTTCCG CGCACATTTC CCCGAAAAGT GCCACCTGAC GTCTAAGAAA CCATTATTAT
9540

CATGACATTA ACCTATAAAA ATAGGCGTAT CACGAGGCC CTTCGTCTTC AA
9592

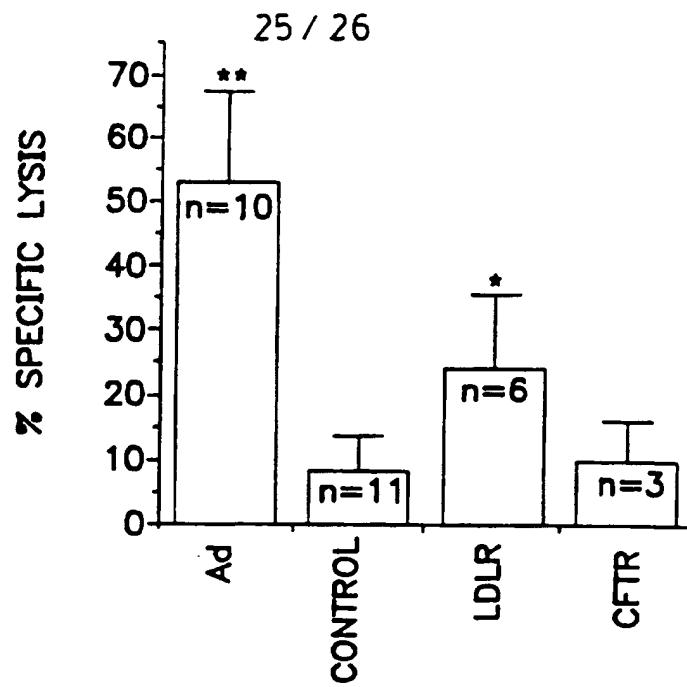


FIG. IOA

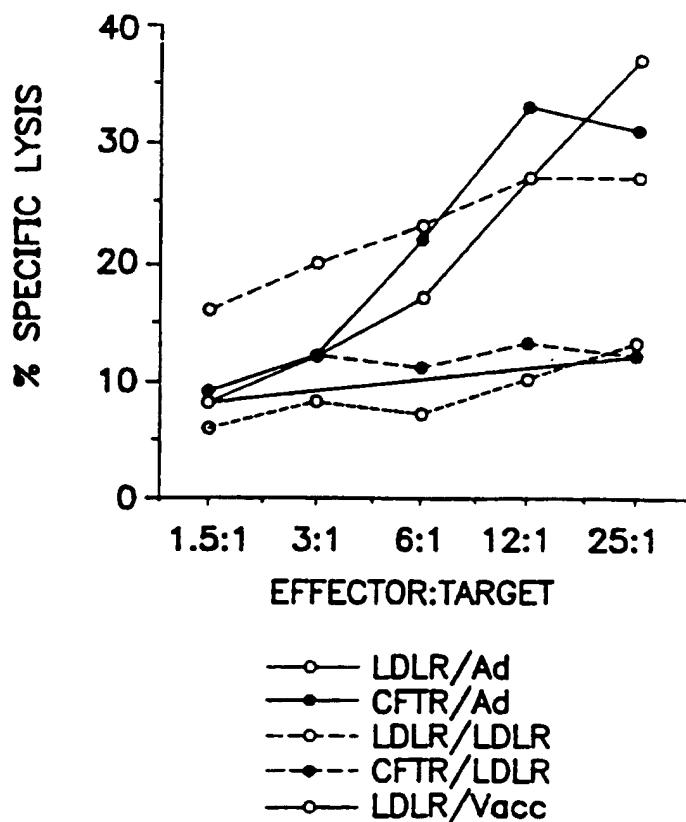


FIG. IOB

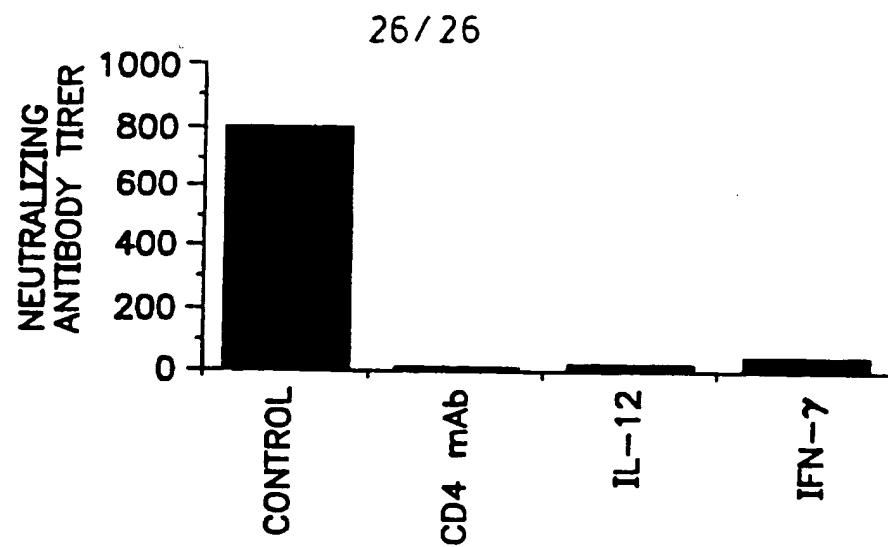


FIG. II A

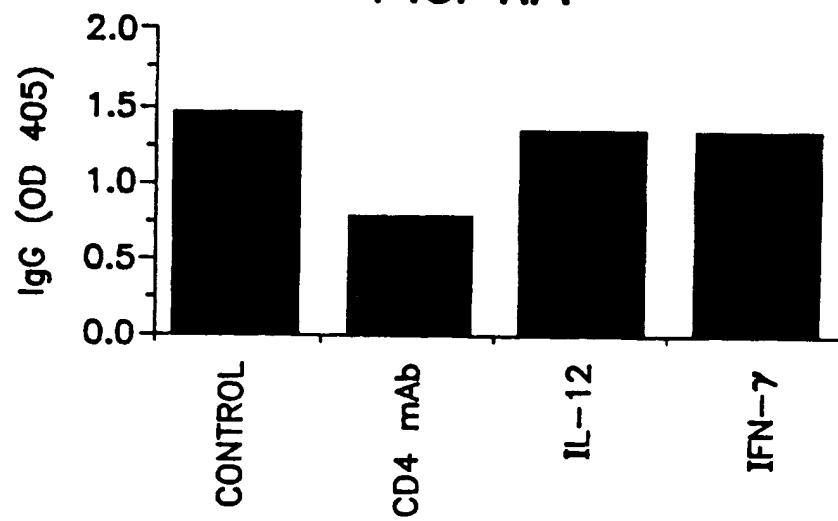


FIG. II B

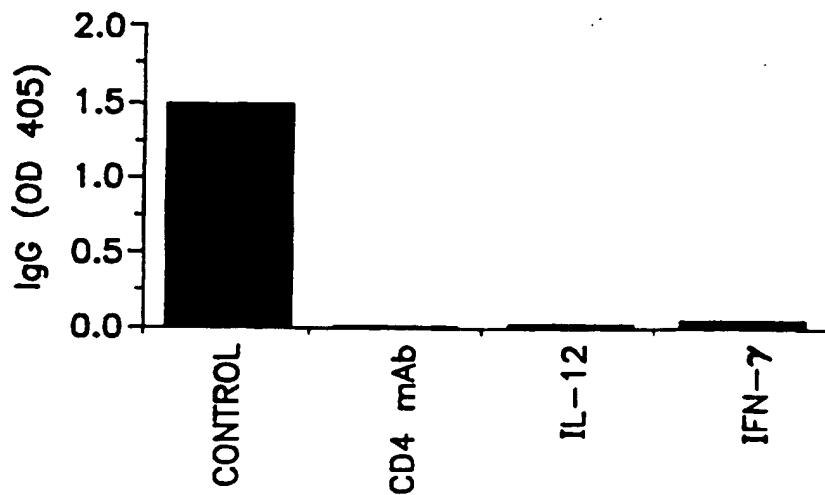


FIG. II C

INTERNATIONAL SEARCH REPORT

International application No

PCT/US 96/03041

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/86 C12N5/10 A61K38/17 //C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 18, 6 May 1994, MD US, pages 13695-13702, XP002007097 K.F.KOZARSKY ET AL.: "In vivo correction of low density lipoprotein receptor deficiency in the Watanabe heritable hyperlipidemic rabbit with recombinant adenoviruses" cited in the application see the whole document --- -/-	1-9

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

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1

Date of the actual completion of the international search

1 July 1996

Date of mailing of the international search report

25.07.96

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/03041

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>THE JOURNAL OF CLINICAL INVESTIGATION, vol. 92, no. 2, August 1993, pages 883-893, XP000574730 S.ISHIBASHI ET AL.: "Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery" cited in the application</p> <p>---</p>	1-9
A	<p>SOMATIC CELL AND MOLECULAR GENETICS, vol. 19, no. 6, November 1993, pages 557-569, XP000574726 M-E-GAFVELS ET AL. : "Cloning of a cDNA encoding a putative human very low density lipoprotein/apolipoprotein E receptor and assignment of the gene to chromosome 9pter-p23" cited in the application see the whole document</p> <p>---</p>	1-9
A	<p>HUMAN MOLECULAR GENETICS, vol. 3, no. 4, April 1994, OXFORD GB, pages 531-537, XP002007098 J.C.WEBB ET AL.: "Characterization and tissue-specific expression of the human 'very low density lipoprotein (VLDL) receptor' mRNA" see the whole document</p> <p>-----</p>	1-9